Osteocalcin and Parental Perceptions

Childhood osteocalcin and its association to early growth, body composition and neurodevelopment, along with parental perceptions on childhood overweight and obesity

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Cover illustration: DNA illustration by Henrietta Nyvang. Graph illustrating the natural pattern of osteocalcin during the first five years of life and bone by Sara Berggren.

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Children have the right to enjoy the highest attainable standard of health
Convention on the Rights of the Child, Article 24

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ABSTRACT

Background and aims: Early programming mean that environmental factors, hormonal levels and early life experiences, like feeding practices, during the first years of life, may have long-lasting effects on later health. One hormone with programming potential is osteocalcin. Early osteocalcin levels might impact later growth, body composition and cognition, but little is known about this hormones' actions and levels during childhood. In adults, low osteocalcin levels are associated to a high body mass index (BMI) and lower cognition. Given that childhood obesity is an increasing public health problem, knowledge of pathophysiology needs to improve, but also knowledge about barriers and facilitators in effective weight treatment. The aim of this thesis was to explore parental perceptions of child weight status, and to increase knowledge about osteocalcin and its potential association to early growth and neurodevelopment.

Methods: This study is based on two longitudinal birth cohorts from Halland, Sweden. The first, which started in 2007, included 2,666 children, monitored with anthropometric data and questionnaires until five years of age (y). The second included 551 children, born 2008-2012 who attended similar follow-ups, but with additional regular blood sampling for 5 years. Of the 551 children, 224 underwent dual energy x-ray absorptiometry (DXA) at age 8 for body composition assessment, and 158 children underwent neurocognitive testing at age 4.

Results: In total, 96.4% respective. 87.1% of parents of two- and five-year-old children with overweight or obesity perceived their child's weight to be just about right. Reference limits for total serum osteocalcin were established. Age

and sex differences were found and the levels correlated to feeding practices, and gestational age. Serum levels of osteocalcin correlated to growth and body composition in a negative way during infancy, but in a positive way during childhood. Osteocalcin at five years of age correlated positively to fat mass index for girls at age eight and to fat-free mass index for boys at age eight. Osteocalcin at four months of age correlated to full-scale intelligence quotient and motor development at four years of age.

Conclusions: Early osteocalcin levels carry information about future growth, body composition and neurodevelopment and may therefore be of importance within the concept of early programming. Osteocalcin shows age and sex-related differences that may be of importance in the development of childhood obesity. The low agreement between parental perceptions of child weight and actual child weight status may be another factor that impacts the development and treatment of childhood obesity.

Keywords: body composition, childhood, early programming, growth, infancy, neurodevelopment, obesity, osteocalcin, perception, sex differences

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SAMMANFATTNING PÅ SVENSKA

Under livets första år sker en snabb utveckling av kropp och hjärna. Tillgång på näring påverkar utvecklingen och hormonnivåer ställer in sig så att kroppen hittar en ”normal” nivå för den aktuella individen. Detta får betydelse för senare hälsa då kroppen programmeras på ett sätt som kan öka respektive minska benägenheten att utveckla ex. obesitas. Studier visar att osteocalcin påverkar kroppsammansättning, minnesförmåga och ångestnivån hos möss, men osteocalcin är även kopplat till lägre kognitiv förmåga, insulinkänslighet och BMI hos vuxna. Kunskap om barn saknas. Då obesitas är ett växande problem, behöver kunskap kring dess utveckling öka men även kunskap kring vad som hindrar och underlättar viktbehandling. Interventioner för att bromsa utvecklingen av obesitas är mest framgångsrika om de utförs tidigt i livet vilket kräver att tillståndet uppmärksammas.

Syftet med doktorandprojektet var att ta reda på om föräldrar noterade när barn drabbades av övervikt, att fastställa referensvärden för osteocalcin hos barn och studera om dessa var kopplade till hjärnans utveckling eller senare kroppsammansättning. Arbetet omfattar fyra studier där vi med hjälp av Halländska födelsekohorter, följt barn från födseln 2007-12 och under uppväxtåren avseende tillväxt, enkätdata, blodprover, DXA (dual energy x-ray absorptiometri) samt utvecklingsbedömning. Den första studien visade att 87% av föräldrar till femåriga barn med övervikt eller obesitas ansåg att deras barn vägde lagom mycket, siffran för två-åringar var 96%. Föräldrar som själva hade övervikt eller låg utbildningsnivå svarade ”lagom” i förhållande till ”för mycket” i högre utsträckning jämfört med övriga föräldrar. Härefter presenterade vi referensvärden för osteocalcin i blodet vid specifika tidpunkter från födseln till fem års ålder. Vi visade att osteocalcin uppvisade könsskillnader och följde ett specifikt mönster med en kraftig topp under första halvåret för att sedan sjunka innan en ny stegring sågs vid 5 års ålder. Våra resultat visade även att amning höjde osteocalcin-värdet under perioden som amningen pågick, och att födelsesätt samt huruvida man föddes stor eller liten för tiden korrelerade till nivån senare i livet. I nästa arbete visade vi att osteocalcin nivån vid fyra månaders ålder uppvisade en negativ korrelation till tillväxt under livets första år men en positiv korrelation för senare värden och tillväxt. Vid fem års ålder korrelerade flickors osteocalcin värden till fettmassa vi 8 års ålder men till fettfri-massa hos pojkar. I den avslutande studien framkom att osteocalcin nivån vid 4 månaders ålder var kopplat till högre IQ och en mer mogen motorisk utveckling vid 4 års ålder.
Sammanfattningsvis bidrar det här doktorandarbetet till ökad kunskap om föräldrars uppfattningar om övervikt och obesitas i barndomen samt om osteocalcin i relation till senare kroppsammansättning och kognitiv utveckling. Genom att lära oss mer om hur barn växer och utvecklas främjas möjligheter till rådgivning och interventioner som på sikt kan bidra till förbättrad folkhälsa.

LIST OF PAPERS

This thesis is based on four studies which have been published, or submitted for publication, in the form of the following four papers, referred to in the text by their Roman numerals.

I. **Berggren S**, Roswall J, Alm B, Bergman S, Dahlgren J, Almquist-Tangen G. Parents with overweight children two and five years of age did not perceive them as weighing too much. *Acta Paediatrica 2017; 107(6):1060-4.*

II. **Berggren S**, Dahlgren J, Andersson O, Bergman S, Roswall J. Reference limits for osteocalcin in infancy and early childhood: A longitudinal birth cohort study. *Clinical Endocrinology 2024; 100(4):399-407.*

III. **Berggren S**, Andersson O, Dahlgren J, Roswall J. Osteocalcin in infancy and early childhood correlates to growth and body composition: A longitudinal birth cohort study. *In manuscript*

IV. **Berggren S**, Andersson O, Hellström-Westas L, Dahlgren J, Roswall J. Serum osteocalcin levels at 4 months of age were associated with neurodevelopment at 4 years of age in term-born children. *Acta Paediatrica 2022; 111(2):338-45.*

ADDITIONAL PUBLICATIONS BY THE AUTHOR

Berggren S, Tiderius CJ. The Cedell method (cerclage wire and staple) leads to less reoperations than the AO method: a retrospective comparative study of 347 lateral ankle fractures. *Acta Orthopaedica*. 2015; 86(3):384-7.

Berggren S, Almquist-Tangen G, Wolfbrandt O, Roswall J. Effects of the COVID-19 pandemic on the physical activity and screen time habits of children aged 11–13 years in Sweden. *Frontiers in Public Health*. 2023; 9(11):1241938.

ABBREVIATIONS

AGA	Appropriate for gestational age
ASQ-III	Ages and Stages Questionnaire, third edition
BDNF	Brain derived neurotropic factor
BGLAP	Bone gamma-carboxyglutamic acid-containing protein
BMI	Body mass index
BMC	Bone mineral content
CHC	Child health center (“BVC” in Swedish)
CI	Confidence interval
CREB	cAMP response element binding
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats / associated system number 9-mediated gene editing technique
DG	Dentate gyrus
DOHaD	Developmental Origins of Health and Disease
DXA	Dual-energy X-ray absorptiometry
FFMI	Fat-free mass index
FMI	Fat mass index
GA	Gestational age
GABA	Gamma-aminobutyric acid
GPR158	the G protein-coupled receptor 158
GPRC6A	G-protein-coupled receptor class C, group 6 subtype A (osteocalcin receptor)
H^2GS	The Halland Health and Growth Study
IGF-1	Insulin-like growth factor 1
IGT	Iowa Gambling Task

IL-6	Interleukin 6
IOTF	International Obesity Task Force
IQ	Intelligence quotient
LDL	Low-density lipoprotein (cholesterol)
LGA	Large for gestational age
MABC-2	Movement assessment battery for children, second edition
MRI	Magnetic resonance imaging
NIHSS	National Institutes of Health Stroke Scale
OC-null	Transgenic mice without the ability to produce osteocalcin
OG	Osteocalcin gene (in mice)
ORG	Osteocalcin related gene (in mice)
OST-PTP	Osteo-testicular receptor-like protein tyrosine phosphatase
RbAp48	histone-binding protein RbAp48 (also known as retinoblastoma binding protein 4, RBBP4)
RCT	Randomized controlled trial
SAT	Subcutaneous adipose tissue
SD	Standard deviation
SDQ	Strengths and Difficulties Questionnaire
SGA	Small for gestational age
vDG	Ventral dentate gyrus
VAT	Visceral adipose tissue
WHtR	Waist to height ratio
WHO	World Health Organization
WPPSI-III	Wechler Preschool and Primary Scale of Intelligence, third edition

DEFINITIONS IN SHORT

Obesity	For adults, obesity is defined as having a BMI >30kg/m^2. For children, age and sex adjusted cut-offs according to IOTF, Cole et al. are used [1].
Overweight	For adults, overweight is defined as having a BMI >25kg/m^2. For children, age and sex adjusted cut-offs according to IOTF, Cole et al. are used [1].

TABLE OF CONTENTS

1 INTRODUCTION

1.1 THE THEORY OF EARLY PROGRAMMING

The theory of early programming, often referred to as "developmental programming" or the "early life origins of health and disease", suggests that environmental factors and experiences during early stages of development can have a profound and lasting impact on an individual's health and well-being throughout their lifespan. This concept is grounded in the idea that critical periods of development, such as pregnancy, infancy, and early childhood, are sensitive to environmental influences that can shape an individual's physiology, metabolism, and risk of various diseases later in life.

Since David Barker and colleagues showed that birthweight correlates to adult death from ischemic heart disease [2], further observations have shown that undernutrition during gestation is a risk factor for adult cardiac and metabolic disorder [3]. This increased risk is thought to be caused by fetal programming that permanently shapes the body, nowadays known as the “Barker hypothesis” [3]. The Barker hypothesis has become the foundation of the science field, today known as the Developmental Origins of Health and Disease (DOHaD) [3]. DOHaD suggests that early life experiences, like inadequate nutrition or adverse conditions during fetal development, can lead to adaptations that increase the risk of chronic diseases, such as heart disease, diabetes, and obesity in adulthood [4]. The DOHaD approach is supported by numerous epidemiological studies, as described in the review by Wadhwa et al. [3]. It relies on empirical observations, environmental clues, and potential epigenetic mechanisms. Early programming may also involve early-life stressors, environmental toxins, and the effect of social determinants of health on developmental trajectories and health outcomes. For instance, children born small for gestational age (SGA) have increased risk for later obesity and children that present with an early adiposity rebound in the preschool years are more prone to developing obesity later in life [5, 6]. However, for children born SGA, an accelerated weight gain during the first months of life is also associated to disturbance in glucose metabolism by the age of 17 [7]. Therefore, the period before birth, early postnatal period and the first years of life may be considered a critical period of life that is especially important for life-long health. However, this period of life also carries opportunities to

prevent future illness through preventive measures such as proper nutrition and health advice [8, 9].

Understanding the concept of early programming has significant implications for public health and preventive medicine. Research in this field has highlighted the importance of maternal health, nutrition, and overall healthcare during pregnancy and early childhood in promoting lifelong health. The concept emphasizes the need for early interventions, to reduce the burden of chronic diseases later in life [10], and serves as the groundwork for this PhD project.

1.2 NEURODEVELOPMENT

Childhood growth and development includes embryonic and fetal neurogenesis. During fetal development, billions of neurons proliferate and migrate to precise locations, which establishes the structural framework of the brain. Simultaneously, synaptogenesis, the formation of connections between neurons, takes place in an excessive manner. After birth, myelination, dendritic arborization and selective pruning continues for three to five years, meaning that there is a protracted period of time for neurodevelopmental maturation. For instance, synapses undergo refinement, called selective pruning, meaning that synapses that are used are strengthened, whereas unused and excessive synapses diminish.

These stages present several critical windows; for instance neurogenesis in the cortical region of the brain mainly occurs during fetal life, and albeit neurogenesis continue to occur later in life, the pace and extent is not comparable to fetal life neurogenesis [11]. Yet, myelination, dendritic arborization and selective pruning continue after birth, giving time for neurodevelopmental maturation. This activity-dependent maturation means that neurons that are not used are lost forever. One example of this activity-dependent maturation is congenital cataract. Because of synaptic pruning and lack of neural circuitry between the eye and brain, these children experience visual impairment, even if the lens is changed later in life. Although newer evidence suggests that white matter plasticity may recover some function in late-visual pathways, vision is not normalized [12]. Therefore, early experiences, sensory input, and environmental stimuli have a profound impact on neural development during these years. Critical milestones, such as language acquisition, motor skill development, and social cognition, are achieved during this phase and nutritional adequacy, play and a caregiving

environment play important roles for optimal neurodevelopment, while neglect or malnutrition during this period can have detrimental and lasting consequences.

Nutrition is known to be important for optimal neurodevelopment [13-17], and deficiencies that occur early in life have time-specific effects. Nutrition may also impact neurodevelopment thorough hormonal actions, an example being iodine deficiency that causes hypothyroidism [18]. However, it is not only nutritional deficiencies but also excess of energy that may impact brain health. Obesity during childhood has been linked to poorer cognitive performance [19] and especially poorer executive function [20]. The relationship is possibly bidirectional but the mechanisms are still unknown. For instance, Guxen et al. [21] showed that low scores of executive function and verbal abilities at age four predicted a high body mass index (BMI) at age six. A large Israeli study on more than two million adolescents showed that individuals with obesity had increased odds for lower cognitive performance and the inability to fully achieve cognitive potential [22].

1.3 EARLY GROWTH PATTERNS

Beside neurodevelopment, the body also undergoes a series of growth stages and spurts, as described by the Infancy-Childhood-Puberty growth model by Karlberg et al. [23], illustrated in Figure 1. These spurts are characterized by sudden increases in height and weight and controlled by various hormones, nutrition and general health status. In infancy, growth is mainly driven by nutritional factors, but hormones like insulin-like growth factor 1, (IGF-1) also play important roles [24]. During the first year of life, infants may triple their birth weight and increase their length by about 50%. In infancy, it is common that children cross height centiles and they tend to find their own curve only after their first birthday. The rapid growth rate seen during infancy slows down during childhood and Growth Hormone (GH) becomes more important while nutrition has less influence. From the age of two, it becomes more uncommon to cross centiles and children usually grow 6-8cm/year and GH may stimulate growth, even without IGF-1 [25]. In childhood, sex differences exist, but they are small, and boys and girls seemingly grow similarly. Later, by the age of six to eight, adrenarche occurs and anabolic hormones are released, which results in a small growth spurt. Lastly, puberty, with its intense growth spurt, occurs, with peak mean height velocity at 12 years for girls and 14 years for boys. During this phase, hormones and nutrition are essential for physical growth. The importance of a balanced diet, including enough but not too many calories,

and sufficient levels of all necessary nutrients, like calcium and vitamin D, are crucial for growth and bone health [26].

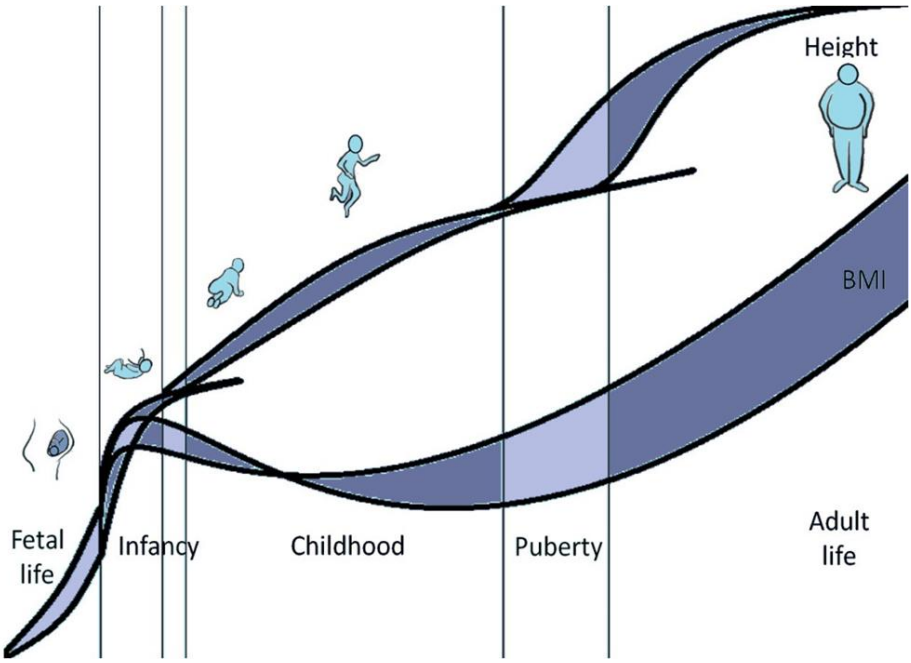

Figure 1, Schematic illustration of the different growth stages. The top graph illustrates height development and the lower BMI. Figure was created by and used with permission from Josefine Roswall.

1.3.1 BODY COMPOSITION

During the various growth stages, the body undergoes significant changes in both height and body composition [23]. Height and weight development is not parallel, as is expressed on the BMI curve, see Figure 2 [27]. Body composition changes significantly during different phases of childhood, and sex differences occur early [23].

During fetal development, adipose tissue starts forming during the second trimester and further accumulates during the third [28]. Therefore, children born preterm have an unproportionally high lean mass (muscle, bones and organs). Normally, preadipocytes, precursors of adipocytes, proliferate and differentiate before birth, based on both genetic factors and maternal nutrition

and health [28]. Adipose tissue is required for energy storage, thermoregulation, as a precursor for hormones and for building the structural part of the brain [29, 30]. In fetal development, adipose tissue primarily accumulates around vital organs, where it isolates and serves as an energy source [29]. High levels of blood sugar speed up the process of fat accumulation and children born to mothers with dysregulated type 2 diabetes are therefore born with large amount of adipose tissue [28]. The phenomenon where carbohydrates result in increased adipose tissue is seen in all infants after birth, thanks to carbohydrate-rich milk or formula given to the child [29].

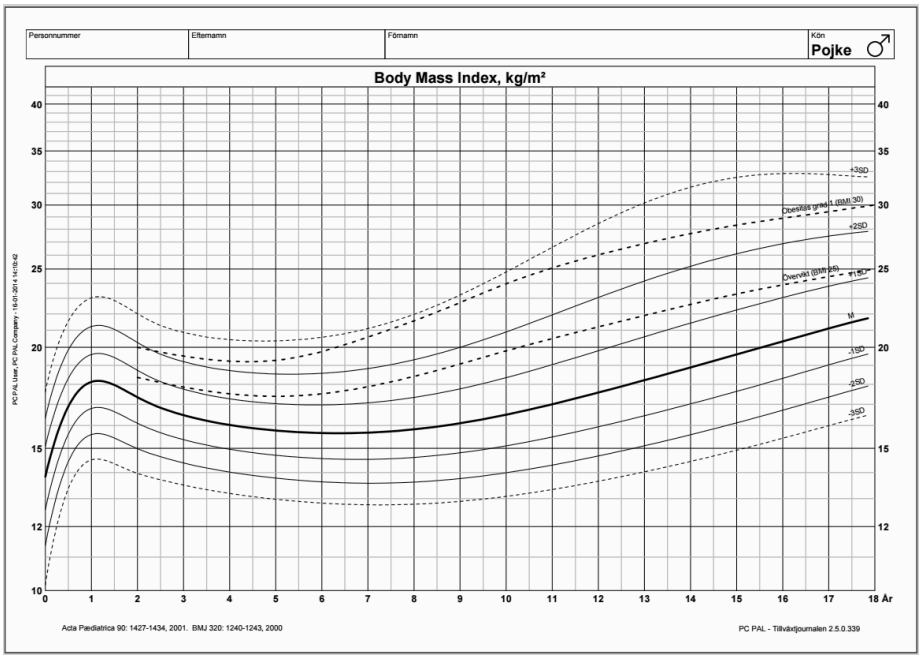

Figure 2, The BMI curve for boys 0-18 years of age based on 1974 Gothenburg men, Acta Paediatrica 90: 1427-1434, with cut-offs for obesity and overweight inserted in accordance with Cole et al., BMJ 320: 1240-143, 200, as used in "Tillväxtjournalen", created by PC-PAL and used in most Swedish medical record systems, including all CHCs in the Halland region at time. Figure used with permission from PC-PAL.

The rapid accumulation of adipose tissue during infancy is evident on the BMI curve, Figure 2, and this energy storage seems to be an evolutionary phenomenon, preparing the child for weaning and learning to eat solid foods [29]. During infancy, adipose tissue continues to expand until the infancy BMI-peak is reached, Figure 3. Thereafter, children become thinner and lean mass

increases during childhood [23]. During middle childhood, somewhere around six to eight years of age most children go through the period known as adiposity rebound. The adiposity rebound is the nadir on the BMI curve, when body fat levels have reached their lowest point and the body starts to accumulate more fat again [6]. The adiposity rebound is another evolutionary important stage, based on genetic and environmental factors, which prepares the body for puberty growth.

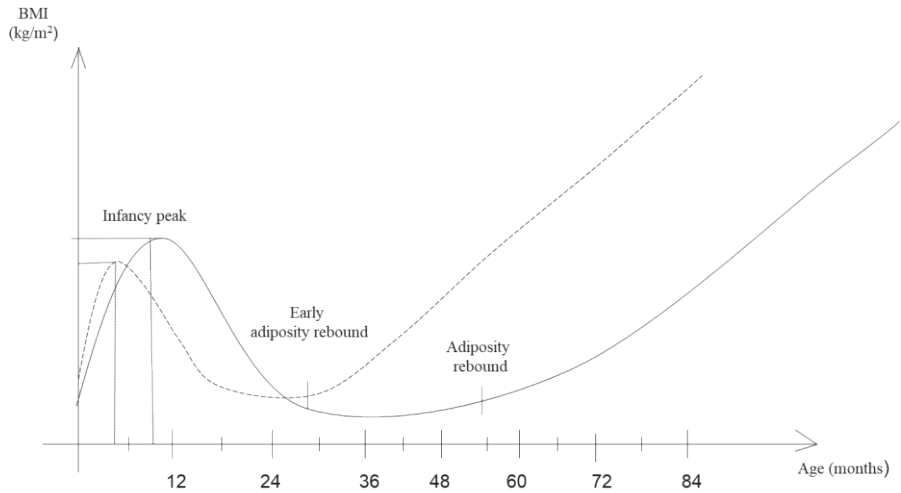

Figure 3, Graphs illustrating BMI curves with two different growth velocity patterns during the first years of life. The dotted line shows an increased pace, with an early infancy peak and an early adiposity rebound compared to more regular timings as shown with the solid line. Figure was created by and used with permission from Josefine Roswall.

The timing of the adiposity rebound seems to be of importance for later health. Research has shown that children who later develop obesity often grow at an accelerated pace, with an early infancy peak and or an early adiposity rebound [6]. If the adiposity rebound occurs before the age of five and half, that is a strong metabolic risk factor associated with higher adiposity level as adults [6, 31]. Furthermore, a heavy body may also hinder natural motor development, with consequences for coordination and bone health [32]. Because of these specific growth phases and developmental stages, the individual growth pattern lays the foundation for future health.

1.3.2 MEASURING BODY COMPOSITION

The modern way of measuring Body Mass Index, BMI, was proposed by Ancel Keys in 1972 [33]. BMI is the weight in kilograms, divided by square height in meters. BMI does not reflect body fat distribution, but is a simple and widely used tool, with high specificity for increased body fat percentage [34]. A general rule of thumb is that a BMI below 17.5 is regarded as thinness, above 25 equals overweight and a BMI above 30 equals to obesity. These cut-offs, and are set to reflect when health risks apply for some adult populations. However, given that children's body composition differs with age, BMI should not be used in the same way for children. In 2000, the International Obesity Task Force (IOTF) proposed the first BMI cut-offs for children aged two and older [35], based on adult BMI cut-offs, which have been updated with charts showing different populations and with sex-specific standard deviation (SD) scores and centiles [36, 37], which were most recently updated in 2012 [1]. Today, BMI is likely the most widely used way to measure body composition, because of its simplicity and relatively long tradition. Comparison between populations is easy and no expensive tools are needed. However, BMI does not reflect body fat distribution and, with increasing evidence that visceral adipose tissue (VAT) is a larger risk factor than both subcutaneous adipose tissue (SAT) and total body fat, waist circumference has been suggested to be a more accurate marker of health risks related to overweight and obesity [38].

To measure waist circumference, a measuring tape is the only thing needed and it is thereby a quick, inexpensive and relatively easy way to assess body composition. Waist circumference better reflects the abdominal fat associated with type 2 diabetes and metabolic syndrome for adults [39-42], but this also seems to be the case for children [43, 44] and adolescents [45]. Waist circumference is dependent on overall size, which is why charts for interpretation are required, especially during childhood years. Adjusting waist circumference with height, by using Waist to Height Ratio (WHtR) has become a useful tool to identify children with high metabolic risk [44, 46]. In adjusting waist circumference with height, a simple cut-off of 0.5 may be used for adults, and children older than five years of age [47]. But, for children younger than five, a more exact number is needed, which is why tables are needed for accurate interpretation [48]. Although both BMI and WHtR are globally used, WHtR is yet not as widespread as BMI [49-51]. Nevertheless, WHtR may tell a slightly different story and may possibly be a more accurate tool for finding risk factors for metabolic health problems.

For more precise measurements of body composition compared to BMI and WHtR, dual-energy X-ray absorptiometry scans (DXA) is considered the gold standard [52]. DXA is an X-ray method for assessing bone mineral density or body composition that utilizes two x-ray beams with different energy levels with low radiation (compared to other X-ray techniques). The attenuation of X-rays is based on atomic number, density and thickness of the object being radiated and, by using different energy in the projections, two different tissues can be distinguished by density [53]. When bone mineral content is the question, the dual technique may answer the question as to whether soft or bone tissue is being radiated. Given that density of fat and lean mass differ, a body composition scan with two different energies may distinguish between the two when projected. DXA-scans are more costly than BMI, waist circumference and WHtR measurements, because it requires an x-ray machine and interpretation of data. However, compared to MRI or other x-ray techniques, the method has relatively low radiation, short scan times, a rapid and easy patient set-up, relatively good measurement precision, stable calibration, etc. [54]. Furthermore, it doesn't need any extra shielding, so operators and parents may stay in the room with the child and, given the low radiation dose, it is suitable for growing children. Still, when exact measurements are needed or when differentiation between visceral and subcutaneous fat is sought, MRI is still the method of choice. MRI is, however, costly, requires the child to be still and demands interpretation of large amounts of data, which makes it inefficient when large cohorts are studied.

1.3.3 ADIPOSE TISSUE

Adipose tissue may be divided into white and brown fat mass. White adipose tissue is involved in obesity, while brown adipose tissue is thermoregulatory and may increase energy expenditure, thereby protecting against fat accumulation in white adipose tissue [55]. Adipose tissue also acts as an endocrine organ, producing hormones and cytokines. Adipocytes (fat cells) produce adiponectin and leptin, while macrophages or stromal cells produce other adipokines [56]. Adipose tissue is also a major site for the metabolism of sex steroids [56].

White adipose tissue grows by hyperplasia and hypertrophy, meaning that adipocytes increase in number and size. If this white adipose tissue increases beyond its capacity, this induces inflammation by macrophage accumulation and inflammatory pathways in the adipose tissue, which is known as lipo-

toxicity and which is thought to have several negative health consequences [57].

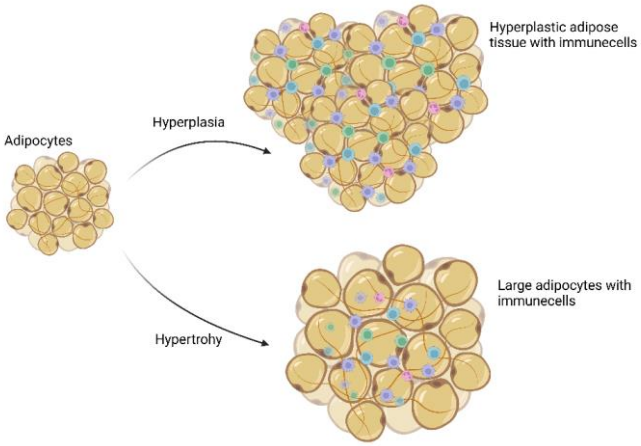

Figure 4, Figure showing the growth of adipose tissue through hyperplasia and hypertrophy. Figure created in BioRender. Berggren,S.(2024)BioRender.com/l66a214

For children, over-nutrition mainly leads to growth of the subcutaneous adipose tissue (SAT), and to a lesser extent growth of adipose tissue around organs, known as visceral fat or visceral adipose tissue (VAT). However, even though subcutaneous fat contributes to the majority (90%) of total body fat by the age of five, visceral fat seems to be more strongly related to metabolic risk than are subcutaneous or total body fat [58]. It is mainly VAT, rather than SAT, that is associated to insulin resistance, type 2 diabetes, hypertension, dyslipidaemia, metabolic syndrome and increased risk for cardiovascular disease [39, 59]. Still, VAT only weakly associates to increased body fat in children and seems to increase only after the subcutaneous fat storage has reached its metabolic capacity [60]. Sex differences apply and VAT increases with increasing age and puberty [61], but because MRI measurements are needed for compartment differentiation, and MRIs are rarely done, due to cost and the need for the child to be still, sometimes requiring sedation at young age, knowledge is limited [62].

1.3.4 CHILDHOOD OVERWEIGHT AND OBESITY

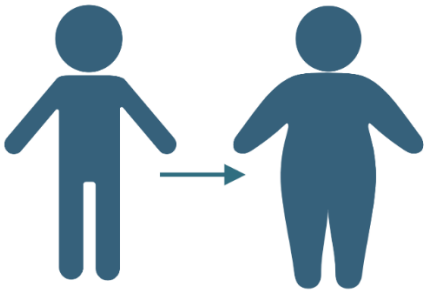

Figure 5, Illustration of a child with normal weight compared to a child with obesity, created in BioRender. Berggren, S. (2024) BioRender.com/t47a959

Overweight and obesity are classified as the largest threat when it comes to non-communicable diseases worldwide, and have thereby become a greater health issue than malnutrition on a global level [32]. The development of childhood obesity is influenced by biological, environmental and social factors that impacts the individual risk for obesity development [63]. In Sweden, about 10% of four-year old children are considered overweight and 2.5% obese [64]. Obesity is a disease known to shorten lifespan [65, 66], while overweight is a risk factor that often leads to obesity [32]. It is well known that obesity is associated to the metabolic syndrome, type 2 diabetes and cardiovascular disease in adults [39, 67-71], but also in children [59, 72]. Besides the risk for early mortality as adults, in childhood, obesity is linked to specific health problems because of the growing body; for instance, obesity in a growing body may lead to orthopedic conditions, increases the risk for asthma and may have psychosocial complications, related to the sensitive period of growing up [32, 72, 73]. Obesity is also linked to lower cognitive performance in children [19, 21], adolescents [22] and adults [74] and is seemingly independent of age [75]. In childhood, obesity is linked to poorer executive functioning [20], while in adulthood, being obese by the age of 40-45 increases the risk for later dementia by 74% [76]. Given that obesity is a condition that impacts the entire body, including the brain, with specific challenges during childhood, prevention and weight management is necessary for future health.

1.4 WEIGHT MANAGEMENT

1.4.1 BARRIERS ON SOCIETAL, HEALTH CARE AND PARENTAL LEVELS

In a society with wide availability of high energy foods, it is comparably easy to become obese, and difficult to regain a healthy weight. Weight management in childhood has the advantage that children rarely need to lose weight, but rather grow into their weight. This may be one reason why it is less difficult to change the trajectory at an earlier stage, compared to later on in life [77]. If treatment is started early, results show a relatively high success rate, and many children will achieve a normal weight that remains over time [77, 78]. Furthermore, childhood treatment is efficient and significantly reduces cardiovascular risk factors over time, compared to adult treatment [32, 72, 79]. Perhaps even more important, since childhood obesity is related to later increased risk for cancer [80-82], even if a normal weight is achieved [72, 80], the shorter the period of over-nutrition will likely lower this increased risk. However, in a clinical experience from the Child Health Center in Halland, Sweden at the beginning of the current century, it was found that parents did not notice when their children became overweight or had obesity. A literature search showed that parents in other parts of the world did not perceive children with overweight as weighing too much [83-87]. Underestimation of weight status among children were also common among the children themselves and among health care professionals [88]. Moreover, parents had a tendency to perceive larger children as healthy, independent of actual weight status [84-86]. At the time, published data were limited on children aged two to five in Sweden. Even though, the study by Regber et al. [83], did include Swedish parents to children aged 2-9, with a mean age of 5.7 years, they did not specify parental perceptions based on child age, but only as one factor of importance for accurate weight perception. Given that the younger years in this interval may be of especial importance for weight management [77] and that an early adiposity rebound, before the age of five and a half, is a risk factor for later obesity [6], we considered the age two to five to be of especial importance. Furthermore, no study with longitudinal data was found, and no study showed whether the parental perception was influenced by regular visits at the CHC.

If parents do not acknowledge overweight or obesity as a health issue, no action will be taken, the child will not receive optimal treatment and the development may proceed without notice [89]. This is a problem, given that studies show that children do not grow out of overweight [90-93], as many parents believe

[94]; rather, overweight increases with age [95]. Additionally, inability to correctly identify overweight in young children may paradoxically lead to overfeeding, related to worrying about the child being underweight [96, 97].

For an intervention to be successful, the problem needs to be addressed and acknowledged as a problem. However, the question of childhood obesity is often regarded by parents as a provocative issue, and studies show that health care professionals deliberately avoid discussing body composition because of the perception of its provocative nature [98]. Furthermore, in North-America and on societal level, obesity is associated to shame and prejudice induced by weight stigma [99]. Weight stigma, together with a lack of training or guidelines of how to communicate about childhood obesity [100, 101] are therefore potential barriers for health professionals to discuss the topic [98]. If nurses do not address overweight and obesity when they spot it, and if parents do not identify or perceive overweight and obesity as a problem, early interventions will not be initiated. Parental perceptions and weight stigma, are therefore possible barriers to weight management, that may impact both the development and treatment of obesity. For this reason, we decided to ask Swedish parents whether they thought that their child weighed too little, just about enough or too much at age two and then again at the age of five and compare this information with actual weight status according to charts.

1.4.2 BARRIERS ON THE INDIVIDUAL LEVEL

Today, it is easier to prevent obesity than to treat it, but what causes children to become obese? We know that obesity is always caused by an excess of calories compared to bodily needs. However, genetic and environmental factors and early life experiences all contribute to the likelihood that an individual develops obesity. Humans have varying levels of vulnerability to an excess of calories, and the probability that one becomes affected is unevenly distributed in the population.

When it comes to the theory of early programming, early life experiences, like being born SGA, increases the risk for later obesity [5]. These children seem to have a physiological memory of nutritional deprivation that makes them energy-frugal. Contrarily, children born to mothers with dysregulated diabetes during pregnancy often become large for gestational age, related to high blood sugar during gestation. These children, however, are not just large at birth; they also have an increased risk for later adiposity. These early experiences of nutritional deprivation or overload becomes a stimuli that during specific critical periods modifies organ function which remains long after the event has

ceased [2]. In mice studies, pups suckling from mothers digesting a high fat diet showed larger leptin surges and became larger during the suckling period, compared to littermates suckling milk from dams receiving regular chow [102]. However, despite being given regular chow after weaning, these pups still presented with leptin resistance in the arcuate nucleus during childhood, despite normal leptin levels [103]. Furthermore, these pups rapidly developed obesity and insulin resistance in response to high fat diet later in life, compared to littermates [103]. Even though there are differences between mice and humans, this highlights that a lot is yet to be learned about the influence of early nutrition and hormonal levels in later obesity. Furthermore, this study proposes one reason to why some individuals are more susceptible to obesity than others [103].

From an evolutionary perspective, preventing starvation is a necessity, but what makes us feel hunger, satiety or drives our foraging behaviour? Children born with leptin deficiency have a strong food searching behaviour and develop morbid obesity at an early age, due to increased hunger and inability to reach satiety. This rare condition does not explain the increasing incidence of obesity worldwide, but it highlights the importance of endocrine signalling in the development of obesity. Leptin is also interesting, because it is produced by adipocytes, and leptin levels normally show a linear association with fat mass in adults [104]. So, adults with obesity have high leptin levels with leptin resistance, and people with leptin deficiency also present with obesity, which highlights the complexity of endocrine signalling even more. Furthermore, leptin may reduce osteocalcin signalling via brain pathways [105]. A lot is yet to be learned about the chemistry that drives our eating behaviours and energy expenditure, but these examples show that obesity is not just a question of waist circumference, but an intricate cavalcade of hormones and neurotransmission. To understand factors behind the development of obesity, research on early life experiences and endocrinological signalling in the body and brain is needed.

1.5 THE HORMONE OSTEOCALCIN

Osteocalcin (also called: bone gamma-carboxyglutamic, acid-containing protein, BGLAP, bone GLA protein or BGP), a hormone produced by the skeleton, might have a regulatory role in the development of obesity and metabolic syndrome, and it might also impact cognition and have reproductive functions [106-109]. Historically, osteocalcin was solely known as a protein involved in bone formation and remodelling [110]. But, when in 1996 osteocalcin-deficient mice were generated by the lab of Ducy et al.,

their phenotype showed no indication of bone pathology, but they had an excess amount of visceral fat [111, 112]. This study led them and the Karsenty et al. lab to investigate correlations between leptin and osteocalcin. In 2000, they demonstrated that leptin was a regulator of bone formation through hypothalamic central regulation, and they proposed a link between bone mass, body weight and gonadal function [105]. The lab continued their work and, in 2007 [113], they showed that osteocalcin-null mice not only had abnormal amounts of visceral fat, but also showed glucose intolerance and insulin resistance, compared to wild-type mice. Additionally, they showed that beta islet size and number in the pancreas were decreased, and concluded that osteocalcin is a hormone that has effects on several organs [113].

Figure 6, Humorous illustration intended to remind the reader that there are biological differences between animals and humans, created in BioRender. Berggren, S. (2024) BioRender.com/h88b177

Of course, there are biological differences between humans and mice [114]. So far, only one gene, the bone gamma-carboxyglutamic acid-containing protein (BGLAP), which encodes osteocalcin, is known in humans [115], whereas mice have three osteocalcin-related (OC) genes [116]. In mice, two of the OC genes, osteocalcin gene 1 and 2 (OG 1 and OG 2, also called bglap1 and bglap2), are expressed in bone, and the other, osteocalcin related gene (ORG), is expressed in the kidney, where it functions in calcium reabsorption [116]. In the experiments on mice by Karsenty et al., osteocalcin has been removed from bone and serum through homologous recombination that removes the OG1 and OG2 in the OC-null mice [111]. ORG in the kidney has not been removed, but its effects are likely negligeable. However, when Moriishi et al. [117] and Diegel et al. [118] produced OC-null mice through the Clustered regularly interspaced short palindromic repeats / associated system number 9- (CRISPR/Cas9) mediated gene editing technique, giving a double knockout effect on the same OG 1 and OG 2 genes, they found opposing results, compared to Karsenty et al. For instance, the genetically modified mice by Diegel et al. did not have significant endocrinological differences, compared to their wild-type littermates [118]. Diegel et al. therefore proposed that

differences between the knockout models may be due to the transcription of neighbouring genes, differences in genetic background or environment.

We have not heard the last word on the issue, and when Lambert et al. created a rat strain using the CRISPR/Cas9, they could not find any differences in body weight or body composition; however, they did find differences in glucose homeostasis [119] between these osteocalcin deficient rats and wild-type rats. Rats only have one osteocalcin gene and may thereby be considered somewhat closer to humans, making this experiment of particular interest. Knowledge is still only emerging, and while some researchers have used knock-out mice, some studies focus on the osteocalcin receptor, the G-protein-coupled receptor class C, group 6 subtype A (GPRC6A). The GPRC6A has affinity for several ligands and is located in various organs, meaning that there are many confounding factors. While the lab by Pi et al. [120] and Oury et al. [121] consistently have shown differences in body weight and glucose homeostasis, Jørgensen et al. [122] showed no differences in body weight or glucose homeostasis, and Wellendorph et al. [123] showed no differences in body weight or fertility based on receptor experiments.

So, animal experiments show conflicting results, and osteocalcin functions on the molecular level are still unclear. In humans it may be considered established that osteocalcin plays a part in insulin secretion and glucose homeostasis [113], but in what way and to what degree are yet to be studied. Less studied but with some evidence is the association between osteocalcin and, respectively, testosterone secretion [124], brain development and cognition [107], adaptation to exercise [125], among other potential functions [126].

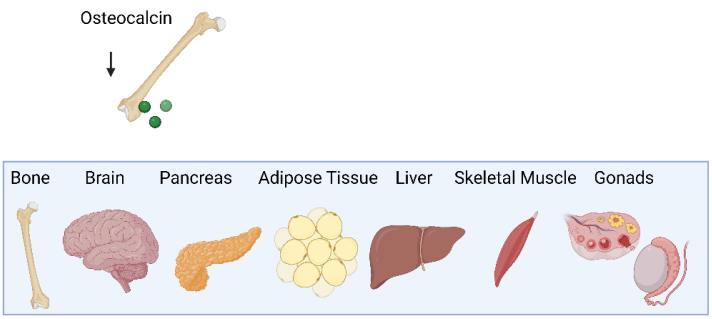

Figure 7, Osteocalcin and some of the proposed target organs relevant for this PhD thesis. Other examples of target organs include: adrenal glands, brown adipose tissue, and blood vessels, interactions with GLP-1 secretion from the small intestine have also been proposed, Figure created BioRender. Berggren, S. (2024) BioRender.com/u05t600

Given this diversity of functions, scientists have tried to summarize the broad functions of osteocalcin in a number of reviews [126-129]. In 2022, Karsenty et al. [126] proposed a theory that osteocalcin functions may represent a physiological response to danger.

The following sections will focus on osteocalcin in relation growth, body composition and neurodevelopment.

1.5.1 MOLECULAR ASPECTS OF OSTEOCALCIN

Osteocalcin is one of the most abundant proteins found in the human body [110, 113, 130]. It is a non-collagenous protein of only 49 amino acids and a molecular weight of approximately 5.8 kDa [110]. Osteocalcin is almost solely produced by osteoblasts. Odontoblasts and some chondrocytes may produce small amounts but the significance on circulating osteocalcin is likely negligible [110]. Osteocalcin is only produced in mineralizing environments and in no other part of the body [110]. The osteoblast produces osteocalcin as a large precursor molecule of 98 amino acids through the BGLAP gene on chromosome 1. This precursor is then cleaved to form the mature protein, carboxylated osteocalcin [131]. However, to become an active protein, it needs to undergo gamma-carboxylation on one to three sites, a process that is dependent on Vitamin K, CO_2, O_2, acidic environment and the enzyme gamma-glutamyl carboxylase. During the carboxylation process, osteocalcin lose its affinity to bone and subsequently enters the circulation as an active hormone, known as undercarboxylated osteocalcin (also referred to as: uncarboxylated osteocalcin or glu-osteocalcin) [132] [113].

The carboxylated osteocalcin also enters the blood stream, but the proportion may vary and the significance of carboxylated osteocalcin in the bloodstream is unknown. In measuring total serum osteocalcin, it is not possible to distinguish between the forms statistically, given the variance in the proportions. It is also important to mention that the amount of osteocalcin measurable in serum thereby does not reflect protein synthesis or the amount of osteocalcin available in the body, but only the amount available in the circulation. The proportion of osteocalcin released to serum may vary with age. Animal studies show that, for young rats <1month of age, <10% is released and >90% is bound to bone, but for adult rats about one third is released to the circulation [110]. Another problem in assessing osteocalcin in serum is the lack of reference values for young children. Attempts to present reference limits have been made, but these often include older children, wide age groups or few children in each age group [133-143].

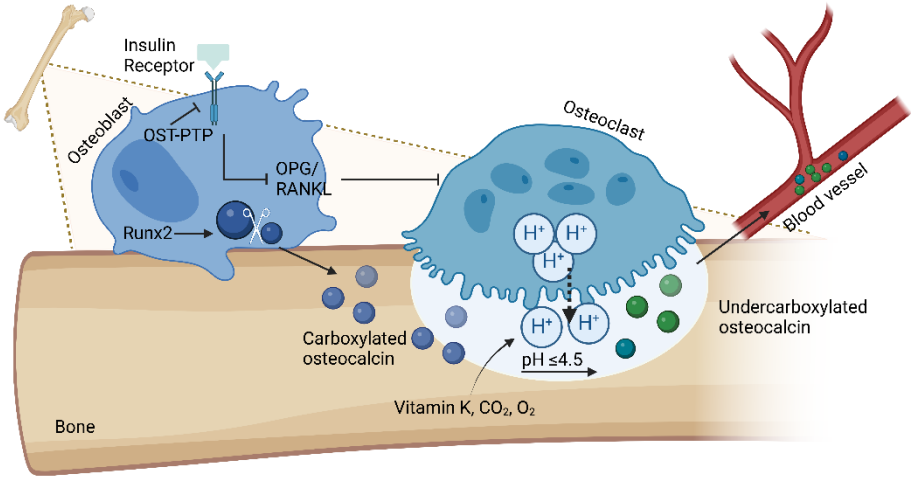

Figure 8, Illustration showing the production of carboxylated osteocalcin in the osteoblast and its way to become an active hormone, known as undercarboxylated osteocalcin (or uncarboxylated osteocalcin). During bone remodeling, osteoclasts resorb calcium and secrete hydrogen ions which give rise to an acidic microclimate in the resorption lacunae. After osteocalcin is produced by the osteoblast, it transports to the resorption lacunae for it to become decarboxylated on one to three glutamic residues, thereby becoming an active protein and losing its affinity to bone (hydroxyapatite), thus enabling entry to the circulation. Figure created Created in BioRender.com/z95h711 based on an original work by Karsenty et al. (83).

1.5.2 OSTEOCALCIN AND BONE

Bone makes up about a fifth of the body weight and has six known functions [126, 144]:

- structural support,
- movement by levers with attached muscle,
- protection for internal organs,
- calcium and phosphorous reservoir,
- blood cell production by the bone marrow,
- hormone production.

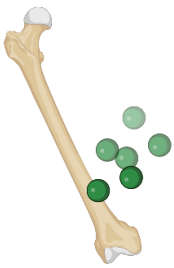

Figure 9, Bone and osteocalcin, Created in BioRender.com/h64l936

Osteocalcin binds to hydroxyapatite, the mineral component of bone, and is thought to regulate bone formation and remodelling, by interaction with

collagen and other proteins in the extracellular matrix of bone. Bone is an organ in constant change, during childhood through bone modelling (longitudinal growth) and after reaching one's final height, bone remodelling. The alteration between bone resorption and formation prevents and heals minimal and major injuries [144], and bone mineralization varies between 0-43% [145]. The rest of bone is organic, with 90% made up of collagen [146, 147] and 10% "non-collagen-proteins" (fibronectin, osteonectin, osteopontin (OPN) and osteocalcin). These proteins influence the quality of bone by collagen accumulation, regulating hydroxyapatite growth, anchoring osteoclasts and more [146]. After the bone has reached full mineralization, which may take decades, no further growth occurs [148]. Bone remodelling is influenced by mechanical pressure and shear stress [149], but also to age, calcium levels and overall health. The constant alteration between bone resorption and formation is dependent on nutrition and to high energetic cost. It is well known that children who do not eat sufficiently will not grow properly and adults who do not eat experience osteopenia or osteoporosis [150, 151]. However, nutritional status alone is not sufficient for optimal mineralization; patients with anorexia nervosa will not restore their bone mineral content solely by resuming regular eating habits [151]. Additionally, starting a moderate energy-restricted diet to induce weight loss will lead to increased bone turnover and lower bone mineral density [152], even with a normal weight. On the other side of the spectrum, patients with obesity will be somewhat protected from bone loss by leptin which inhibits bone resorption via the central nervous system [105]. Having a higher weight seems to protect against low bone mineral density [153]. Nevertheless, obesity seems to be a risk factor for fractures in children [154]. This supports the notion that there is an axis between adipose tissue and bone, where osteocalcin likely plays a part.

1.5.3 OSTEOCALCIN AND BODY COMPOSITION

Since the first study that showed an association between body composition and osteocalcin [111], many more studies have been rolled out. A meta-analysis of 51 human studies in 2020 concluded that osteocalcin shows an inverse relationship to BMI in adults [155]. Similar findings in adolescents and young adults have shown that high osteocalcin in serum correlated to lower BMI, smaller waist circumference and lower systolic blood pressure, along with lower levels of the cholesterol low-density lipoprotein (LDL-C) [156]. Likewise, osteocalcin negatively correlated with adiposity in 11-to-14-year-old American school children [157] and for German children with obesity

[158]. The German study also showed that substantial weight loss over one year led to increased osteocalcin levels and decreased leptin levels [158].

Keeping in mind that body composition is more than BMI and adiposity, for children 7-12 years of age, total osteocalcin was lower for obese children, with a negative correlation towards fat percentage and visceral fat area, but also a positive correlation with lean body mass, fat-free mass and fat-free mass index [159].

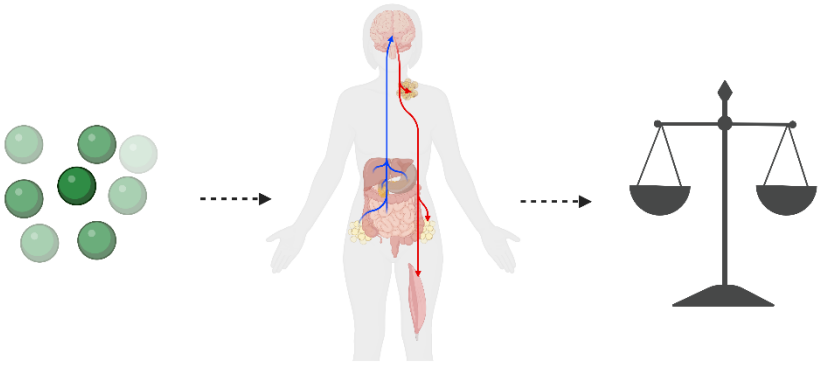

Figure 10, Osteocalcin has many target organs that may impact body composition and weight, of which some are within the metabolic pathways. Created in BioRender. Berggren, S. (2024) BioRender.com/o01e716

Lean body mass includes muscle mass and it is possible that osteocalcin may exert effects on skeletal muscle. Karsenty et al. (124) showed that mice lacking osteocalcin developed deficits in muscle function over time and that osteocalcin was necessary to maintain sufficient muscle mass in older mice. They showed that osteocalcin signaling in myofibers increased insulin-dependent glucose uptake [160] and fatty acid uptake during exercise, and that osteocalcin accounted for the exercise-induced release of interleukin-6 (IL-6) [125] [161]. IL-6, a myokine, believed to be involved in muscle hypertrophy and myogenesis, exerts systemic effects on the gluconeogenesis in the liver and general glucose uptake [162]. Circulating levels of osteocalcin surge after aerobic exercise and seems to be necessary for optimum adaptation to exercise [125].

Even so, it remains unknown whether osteocalcin relates to muscle mass and function, which would impact the interpretation of studies on osteocalcin and body composition. To date, we have only found one study on muscle mass and

osteocalcin in humans [163]. This study did find a positive correlation with lower limb muscle strength for women over the age of 70 and uncarboxylated osteocalcin in relation to total osteocalcin, which could support osteocalcin function in muscle.

Still, little is known in relation to children younger than five years of age, when it comes to osteocalcin in relation to fat mass and lean mass. Although some signalling pathways are known, it is still not known whether early osteocalcin levels reflect body composition either at time or later in life.

1.5.4 OSTEOCALCIN AND ADIPOKINE CROSSTALK

A likely mechanism through which osteocalcin associates to BMI in adolescents and adults is regulation of adipose tissue metabolism. Among other proteins, adipocytes produce the adipokines leptin and adiponectin. Osteocalcin has been shown to stimulate the expression of adiponectin, which in turn regulates energy metabolism and insulin sensitivity. Adiponectin in adults is inversely related to insulin sensitivity and often used as a surrogate marker for insulin resistance. Furthermore, adiponectin also promotes fatty acid oxidation in the liver and skeletal muscle, which can help to prevent the development of obesity and insulin resistance. Polgreen et al. [156] showed that carboxylated osteocalcin correlated directly to adiponectin.

Leptin reduces the production of osteocalcin via the brain and sympathetic nervous system, and osteocalcin increases the expression of leptin in adipocytes [164].

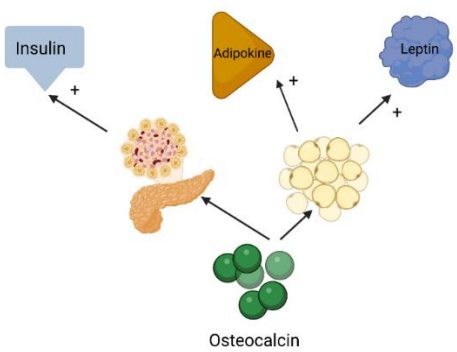

Figure 11, Illustration of osteocalcin signaling with pancreas and adipose tissue as target organs with resulting release of insulin, adiponectin and leptin. Created in BioRender. Berggren, S. (2024) BioRender.com/r71z231

Osteocalcin is involved in both the leptin and insulin signaling pathways and both of these hormones have key roles in the regulation of body weight and energy homeostasis. Patients with obesity frequently develop resistance to both of these hormones. In the brain, the hypothalamic proopiomelanocortin and agouti-related protein neurons have been identified as key targets of leptin and insulin, that may modulate the effects and interpretation of these hormones [165]. Leptin is known as a satiety-regulating hormone which, in its absence, results in severe obesity. From childhood and throughout life, leptin levels correlate to fat mass in a positive way [104], but leptin levels are also subject to sex differences and influenced by sex hormones. Girls have larger fat mass compared to boys, but girls also present with higher leptin levels compared to boys, independent of fat mass [166, 167]. These sex differences may in part be explained by differences in testosterone levels, given that testosterone suppresses leptin levels [167, 168]. This was supported by a study which showed that treating transsexual individuals with cross-sex hormone reversed the sex differences seen for leptin [169]. Leptin is therefore one hormone that is likely closely related to osteocalcin in its actions and effects, and a hormone that is influenced by sex differences, which elucidates a bone-adipose-gonadal-brain-axis.

Another interesting aspect of leptin is that, even given that leptin correlates to fat mass, leptin levels are low in human infancy, the most fat accumulating period of life [170]. This is interesting from a physiological perspective, since mice studies show that leptin-levels during the first two weeks of life do not correlate to body composition or fat mass; rather, they coincide with the development of major hypothalamic feeding circuits [171]. This study from Bouret et al. [171] and the one from Ramos-Lobo et al. [172] show that leptin levels in the neonatal period programs the leptin-set-point, which decides how the mice will interpret leptin signals later in life [172]. Evidence suggests that changes in leptin levels due to nutrition in early life can structurally impact hypothalamic feeding circuits. This is illustrated by the experiments with pups reared on milk from dams receiving a high fat diet, which alters leptin levels and susceptibility to obesity later in life [102].

Leptin is therefore one example of a hormone that may have various different roles and effects during different periods of life, and it is an example of a hormone with possible effects on early programming, along with having a close relation to osteocalcin. Leptin is therefore also an example of why the abovementioned studies on osteocalcin and BMI cannot be extrapolated to younger children.

1.5.5 OSTEOCALCIN AND BRAIN FUNCTION

In 2013, Karsenty et al. [107] showed, in mice, that osteocalcin passed through the placenta to the pup and further through the blood-brain barrier in the offspring, where it prevented neuronal apoptosis in the hippocampus. The study revealed that osteocalcin enhanced spatial learning and memory in mice during embryogenesis. Furthermore, they showed that OC-null mice, who lacked the ability to produce osteocalcin, expressed behavioral traits associated with depression and anxiety and of impaired memory formation and spatial learning [107]; yet, when injected with osteocalcin their behavior normalized. Human studies imply that the hippocampus is involved in spatial cognition, memory and anxiety [173]. Uncarboxylated osteocalcin was then shown to enhance the synthesis of monoamine transmitters (serotonin, norepinephrine, dopamine) and inhibit gamma-aminobutyric acid (GABA) by regulating specific enzymes [107] – all crucial for cognitive function. The finding that osteocalcin associates to cognitive function was strengthened by Khrimian et al. [174], who found that mice with lower uncarboxylated osteocalcin levels showed cognitive impairments and increased anxiety. Furthermore, they showed that, when plasma from young wild-type mice were injected in elderly mice with defects in hippocampal-dependent memory, the elderly mice got enhanced memory function and lowered anxiety levels. However, if the experiment was repeated with plasma from young OC-null mice, no improvements were seen. On a molecular level, the G protein-coupled receptor 158 (GPR158) is the osteocalcin receptor in the brain [175, 176], and osteocalcin signaling through the GPR158 regulates histone-binding protein RbAp48 expression in the hippocampal formation [175]. The RbAp48 in turn impacts histone acetylation, and acts as a regulator on brain derived neurotropic factor (BDNF) transcription, and interacts with the cyclic AMP response element binding (CREB) protein pathway (a cellular transcription factor, controlling expression of various genes). RbAp48 shows age-related decline in the dentate gyrus, which is one molecular mechanism for age-related memory loss [177]. Mice models show that RbAp48 is lower in older mice compared to young mice [175], and specifically lower in the dentate gyrus of older mice. Inhibition of RbAp48 in young mice causes hippocampal-dependent-memory deficits, similar to those related to aging [177].

The dentate gyrus is interesting because it is one area of the hippocampus were adult neurogenesis is possible [178]. The dentate gyrus may be thought of as a gate that controls input to the hippocampus. Research has shown that the dentate gyrus is responsible for spatial learning and memory generation, but also to mood regulation, why the dentate gyrus may be of importance when it comes to depression and anxiety [179]. The dentate gyrus, is therefore interesting from the perspective of osteocalcin, given that it is those areas, of

spatial learning, memory and mood regulation, that OC-null mice showed impairments [107]. Dentate gyrus granule cells seem to control specific features of contextual learning and anxiety [180]. Anacker et al. [181] showed that inhibition of adult-born neurons in the ventral Dentate Gyrus (vDG) promoted susceptibility to social defeat stress, while increasing neurogenesis in this area conferred resilience to chronic stress. Their conclusion was that the vDG might be a key factor in determining individual vulnerability to stress and psychiatric disorders.
Furthermore, osteocalcin is proposed to have an influence on motor function. In rats with induced Parkinson's disease, improvements in cell survival in the substantia nigra, was seen after being injected with osteocalcin and the rats presented with reduced motor deficits [176].

Human observational studies support the association between osteocalcin and brain function. In a study by Puig et al. [109], low serum osteocalcin levels were associated with a higher BMI and lower cognitive performance, and to micro-structural changes in the basal ganglia and hypothalamus of the brain. The study was based on only 24 adults, but still reveals interesting evidence of osteocalcin functions in the brain as measured by the Iowa Gambling Test (IGT-test, a psychological test of decision making). Another study of 196 adult men with type 2 diabetes [108] presented similar associations between low osteocalcin levels and impaired cognition. Wu et al. [182] showed that ischemic stroke patients with better outcomes had higher serum osteocalcin levels than those whose National Institutes of Health Stroke Scale (NIHSS, a tool for quantifying impairments caused by a stroke) scores did not improve.

Nevertheless, if osteocalcin affects brain areas responsible for memory, spatial cognition or motor control, osteocalcin could potentially influence these functions. Given that, significant brain development occurs before birth and during infancy, infant osteocalcin levels are particularly interesting from a developmental perspective.

1.5.6 OSTEOCALCIN, ADRENAL FUNCTION AND GLUCOSE METABOLISM

It would seem remiss to write about osteocalcin without addressing its relation to glucocorticoids or glucose metabolism. Even though it is not the focus of this PhD project, I have decided to dedicate some space to this topic, below.

In 1995, before osteocalcin functions on metabolism were discovered, scientists found that acute stressors, tested by immobilization for rats, raised osteocalcin levels by 50% within 5-20 minutes, together with increased

corticosterone, epinephrine and norepinephrine. Removal of epinephrine did not impact the osteocalcin response, but adrenalectomy showed that corticosterone and norepinephrine exerted a suppressive effect on the osteocalcin response [183]. This was strengthened by Berger et al. in 2019 [184], who showed that osteocalcin suppresses the parasympathetic nervous system, thereby enabling a stress response within minutes after detecting fear.

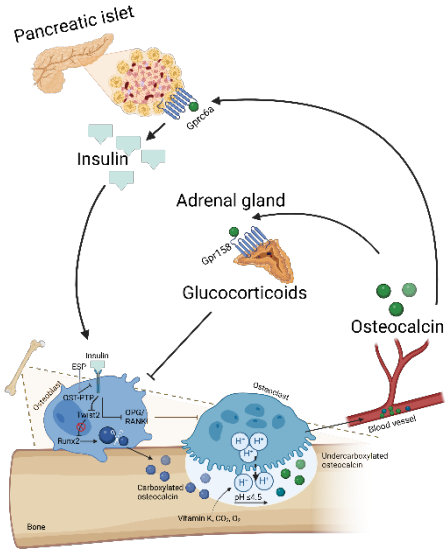

Figure 12, Very simplified graphic illustration of osteocalcin, adrenal function and glucose metabolism, Created in BioRender. Berggren, S. (2024) BioRender.com/d728384

Yadav et al. also proposed that osteocalcin could regulate adrenal steroidogenesis and mineralocorticoid biosynthesis in a classic feedback loop, this through the GPR158, osteocalcin receptor, located in the adrenal cortex [185]. Glucocorticoids appear to reduce osteocalcin activity by inhibiting osteoblast function and the production of osteocalcin [186]. Corticosterone treatment has known side effects, including lowered bone mineral density, increased risk for type 2 diabetes and central adiposity. These side effects are surprisingly similar to phenotypes of osteocalcin-deficient mice. Perhaps osteocalcin may have a role in the stress response [126], and thereby also have a role in the side effects experienced by patients with long-term corticosterone treatment.

Several studies have shown that osteocalcin is involved in the regulation of glucose metabolism; by stimulating insulin secretion from beta cells in the pancreas and by increasing insulin sensitivity in peripheral tissues (like skeletal muscle and adipose tissue) further improving glucose uptake [113, 164, 187, 188]. Although there are conflicting results in animal studies, evidence that osteocalcin impacts human glucose and insulin response was strengthened by a study showing that polymorphism in the GPRC6A gene, which encodes osteocalcin, is related to insulin resistance in humans [189]. In a systematic review and meta-analysis of several human observational studies, it was confirmed that circulating osteocalcin levels are higher in subjects with normal glucose tolerance, compared to adults with type 2 diabetes [190]. Furthermore, cross-sectional studies show, that osteocalcin has inverse associations to dysmetabolic phenotypes, with lower fasting insulin and homeostasis model assessment for insulin resistance (HOMA-IR) [191-193]. Less studied is the association between osteocalcin and glucose metabolism among children, but, for prepubertal children, similar associations between insulin sensitivity and osteocalcin are found among children with overweight [194]. Similarly, Reinehr et al. showed that, for children with obesity, substantial weight loss was associated to increased osteocalcin levels, decreased leptin levels and decreased HOMA-IR [158] – a sign of reduced insulin resistance. The authors therefore proposed that osteocalcin might be a link between obesity and insulin resistance. However, seven years later, Giudici [195] did not find support for this theory in their study on healthy children aged 9-13. And Tubic et al. [106] did not find an association between osteocalcin and metabolic profile among prepubertal children. The reason for these conflicting findings may lie within the age group studied, the mixed sex approach or perhaps exercise level among children or in differing methods of analysing osteocalcin.

1.6 RATIONALE FOR THE THESIS

Children have the right to enjoy the highest attainable standard of health. To achieve this, it is important to maintain a healthy weight status and enable optimal neurodevelopment. Childhood overweight often leads to the disease obesity. Obesity is always caused by excess calories in relation to nutritional needs, but the likelihood that a child will develop obesity differs based on genetic factors, early life experiences and environmental factors [63]. From a health perspective, childhood obesity is associated to specific secondary complications related to the growing body [32, 72], but it is also associated with the same complications seen for adults, including a shorter life span [32, 59]. Given that, without treatment, children do not grow out of obesity [90-93], but, rather increase their level of obesity [95], action needs to be taken.

There are many potential barriers to effective weight treatment, on different levels. On a societal level, barriers may include a general idea that children grow out of overweight [94], or that it is a good thing if children "have a little extra weight" [96, 196]. Barriers on health care level may include difficulties in identification of overweight and obesity [88], or in the communication with parents, given that the question is considered sensitive or provocative [98]. In this PhD thesis, we will focus on parental and child factors that may impact childhood weight management and neurodevelopment.

Research has shown that parents do not acknowledge when children become overweight or obese [83-87]. However, it is not known to what extent this applies in a Swedish setting for children aged two to five, or if visiting the CHC increases the likelihood of parental acknowledgement of the problem. It is also possible that parental perceptions differ based on parental factors, such as educational level, country of birth or parental weight status. At the individual level, early hormonal levels may be factors that increase the likelihood for childhood obesity through early programming. One such hormone is osteocalcin. Osteocalcin may be involved in the development of obesity but it is not known whether osteocalcin in childhood correlates to body composition, at the time or later in life. There is a gap in knowledge and research is hampered by the lack of reference values and the lack of knowledge about factors that impact early osteocalcin levels. Furthermore, growing evidence suggests that there is a link between the development of obesity and worse cognitive functions [19-21]. Osteocalcin may be involved in memory formation and overall cognitive function [107, 126], but there is a gap in knowledge whether these findings apply to children.

There is a need to increase knowledge about factors that may hinder effective weight treatment, like parental perceptions on childhood weight status or hormonal levels, and to increase knowledge about factors that may impact the development of obesity or neurodevelopment, with a view to prevent future childhood obesity, facilitate effective weight treatment and improve overall health.

If we know better, we do better.

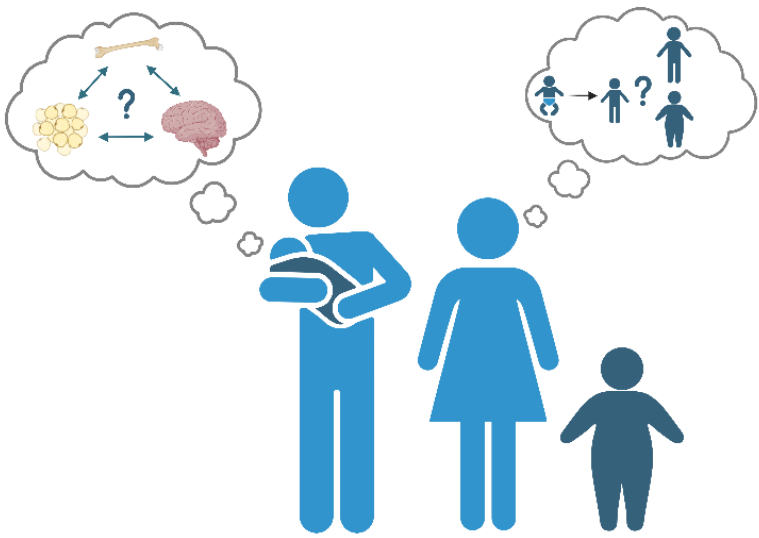

Figure 13, Graphic illustration of a family, showing the rationale of the thesis, created in BioRender. Berggren, S. (2024) BioRender.com/f81m642

2 OVERALL AIM

The aim of this thesis was to explore barriers to effective weight management and to increase knowledge about osteocalcin and its potential association to early growth and neurodevelopment.

2.1 SPECIFIC AIMS

1. To investigate whether parents of preschool-children with overweight or obesity perceive that their children weigh too much, and to explore whether sociodemographic factors influence the agreement between actual and perceived weight status (Paper I).
2. To present reference limits for total serum osteocalcin for specific timepoints from birth until five years of age, and to identify early factors that may influence these levels (Paper II).
3. To investigate if early osteocalcin levels correlate to height, weight or body composition at the time and later in life (Paper III).
4. To investigate whether early levels of osteocalcin correlate to neurological development, measured as intelligence quotient, motor development and behavior at four years of age (Paper IV).

3 METHODS

3.1 STUDY POPULATION

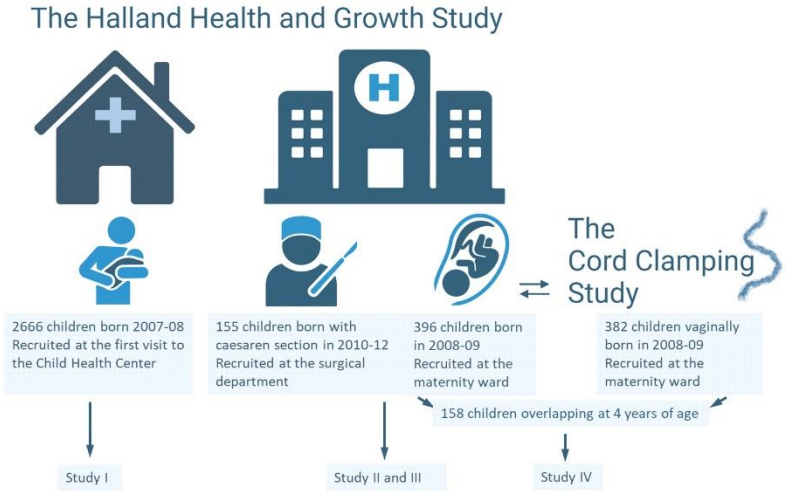

Figure 14, Schematic illustration of the study populations for the different studies included within this PhD thesis, created in BioRender. Berggren, S. (2024) BioRender.com/z47s533

For this PhD project, three cohorts have been used: A large longitudinal birth cohort the Halland Health and Growth Study, H^2GS [197], the Small H^2GS cohort[198], and the randomized controlled trial (RCT) the Cord Clamping Study [199], Figure 14. The H^2GS is a longitudinal birth cohort that invited all children born in Halland, Sweden, from October 2007 to December 2008 (n = 3,860). Parents of 2,666 children (69%) agreed to participate in the study and turned in the signed informed consent form at their first visit to the Clinical Health Center, (CHC, In Swedish: BVC). These 2,666 children serve as the base for the first study about parental perceptions (Study I).

While the H^2GS-study continued, 400 families were recruited upon their arrival at the maternity ward at Halland Hospital, Halmstad to a more comprehensive follow-up program, called the Small H^2GS cohort. Of these, parents of 396 children born April 2008 - June 2009 turned in the informed consent form. These 396 children comprised children born vaginally from gestational week 28+0, and children born by caesarean section (n = 32), but, since most of the mothers with planned caesarean sections entered the hospital through the surgical department, caesarean sections were underrepresented.

Because of this underrepresentation of planned caesarean section, a new inclusion period started at the surgical department with planned caesarean sections between May 2010 and February 2012. Inclusion closed when 155 children had been recruited.

For study II and III, this gives 551 included children (281 boys, 51%) in the Small H^2GS cohort, born between 2008-2012, Figure 14. Of these 551 children, 486 children were born full-term (from 36+6 to 41+6 weeks/days of GA), 33 children were born preterm (from 31+6 to 36+5 weeks/days of GA) and 32 children were born postterm (from 42+0 to 42+4 weeks/days of GA).

In 2008, at the same hospital, another researcher at the same clinic started an RCT, the Cord Clamping Study [199]. Since the Small H^2GS Cohort and the Cord Clamping Study overlapped in time and place, 258 of the 396 children in the Small H^2GS overlapped and the researchers collaborated with blood sampling and follow-ups to minimize the effort needed by the families. Of these 258 children, 158 children were still in both studies at 4 years of age and completed the neurodevelopmental assessment. These 158 children make up the population for Study IV, on osteocalcin and later neurodevelopment. The different study populations are described in more detail in these published articles [198-200].

There were no exclusion criteria for the H^2GS or the small H^2GS Cohort, but parents had to understand Swedish well enough to receive information about the study and provide informed consent. For Study I, families recruited at the CHC included all children registered in Halland and born during the inclusion period, independent of gestational age at birth or where the child was born. But, since recruitment for Study II-III was done at the Halland Hospital Halmstad, children born extremely prematurely (< gestational week 28+0) were practically excluded, because they were transferred to the nearest university hospital. For Study IV, inclusion and exclusion criteria applied; in general, children were vaginally born at full term and had to be healthy, see Anderson et al. [198, 199] for details.

For study II, on reference values, a subgroup of 459 of the 551 subjects was created to provide reference limits for healthy infants and children. These healthy infants were born at full term and born appropriate for gestational age, and no child was born to a mother with diabetes mellitus. For this subgroup, exclusion was applied stepwise, meaning that children could only be excluded once, even if more reasons for exclusion were present (Figure 1, Paper II). Of these 459 children, 142 children were born by caesarean section, of which 32

were included in 2008-2009. To analyze the reference populations' generalizability, the reference population of 459 children was compared to the general population, by using data on all 1,579 full-term children who were born in Halmstad between April 2008 and June 2009, with the reference population subtracted. The data were obtained from the Swedish National Board of Health and Welfare (Table 1, Paper II) [201].

All children born in Sweden today and during the recruitment period are and were invited to the CHC with regular and voluntary visits from birth until five years of age. All visits are free of charge and families meet nurses and physicians for regular check-ups, including vaccinations, screening for visual or hearing deficits and regular anthropometrics. During the recruitment period, about 98-99% of parents of all children born in Sweden agreed to these regular CHC follow-ups. All children born in Halland 2007-2012, independent of study participation, received the following supplementation: Children born full-term or post-term received 1mg/kg of intramuscular vitamin K after birth and 0.4 mg/kg was administered to children born preterm. All the parents were given free vitamin D supplements at the CHC and were advised to give the child 400 IE/daily from two weeks of age until 2 years of age. Depending on gestational age and size for gestational age, additional supplementation (iron, multi-vitamins) were given the first 6 months, in accordance with national recommendations.

3.2 STUDY DESIGN

Table 1, Table showing an overview of the four included studies in this PhD thesis with their study characteristics

	Study I	Study II	Study III	Study IV
Study design	Observational, prospective cohort	Observational, prospective cohort	Observational, prospective cohort	Observational, prospective cohort
Setting	Included at first CHC visit	Included at birth, maternity ward and surgical department	Included at birth, maternity ward and surgical department	Included at the maternity ward
Inclusion criteria	All newborns in the region going to CHC, understand Swedish	All children born at the departments in Halmstad understand Swedish	All children born at the departments in Halmstad understand Swedish	Normal pregnancy, vaginally, term born in Halmstad, Understand Swedish, "Healthy"*
Exclusion	n.a	(Gestational week <28, not born in Halmstad)	(Gestational week <28, not born in Halmstad)	Serious malformations, syndromes or congenital disease*
Nomber of participants	2666	551	551	158
Years of Inclusion	2007-2008	2008-2012	2008-2012	2008-2009
Study outcome	Body composition, parental perceptions	Total serum osteocalcin	Total serum osteocalcin, growth, body composition	Total serum osteocalcin, neurodevelopment
Outcome assessment	BMI, Questionnaire	Total serum osteocalcin, sex, age, questionnaire, food-diary	Anthropometrics, BMI, WHtR, DXA, Total serum osteocalcin	WPPSI-III, SDQ, ASQ-III, MABC-II, Total serum osteocalcin

*See Andersson et al. for details [199]. CHC = clinical health center (Sve: BVC), RCT = randomised controlled trial, BMI = body mass index, WHtR = waist to height ratio, DXA = dual energy X-ray absorptiometry, WPPSI-III = Wechler Preschool and Primary Scale of Intelligence, third edition, SDQ = Strengths and Difficulties Questionnaire, ASQ-III = Ages and Stages Questionnaire, third edition, MABC-2 = Movement Assessment Battery for Children, third edition

STUDY I

For participants in the H²GS study, families received extra questionnaires at the regular check-ups, but beside these questionnaires the check-ups were similar for participants and non-participants, meaning that anthropometrics were gathered for all children but, for participants, these were also noted in the study protocol. The participating parents filled out the questionnaires at the CHC. The first questionnaire included background data, including parental age, country of birth, educational status, smoking habits, parental height and weight, health-status and more. At the two-year and five-year follow-ups, questionnaires including the question "Do you think that your child weighs: Too much, just about right, or too little?" among other questions were handed out. Data were gathered and digitalized, after which statistics were run.

STUDY II-IV

For the Small H²GS cohort, similar background data as in Study I were gathered at the first follow-up, together with information about pregnancy, delivery and information about the first days of life. Birth length, weight and head circumference were gathered from charts. Questions about breast feeding vs. formula feeding were asked at four months of age and a 48-hour food diary was handed out at 12 months. Blood samples from cord, 4, 12, 36 and 60 months of age were sampled and frozen for later analysis. For Study IV, neurodevelopmental assessment was performed at 48-51 months of age.

3.2.1 GROWTH AND BODY COMPOSITION

For Study I, anthropometric measurements, height, weight and waist circumference were gathered at the regular visits at CHC at 4, 6, 12, 18, 24, 36, 48 and 60 months of age. For Study II-IV, height and weight was measured by trained research nurses in a standardised manner at the paediatric clinic at the Halland Hospital Halmstad, Sweden. Follow-ups were also offered at 78 and 96 months of age. In all studies (I-IV), length was measured in supine position for children younger than two and thereafter height was measured with the child standing with heels and shoulder blades touching the wall. Weight was measured naked or in light underwear on calibrated scales, for all studies. Waist circumference was measured midway between the lowest rib and the iliac crest, at the end of a gentle expiration and using a soft measuring tape.

For assessment of body composition, body mass index (BMI), waist to height ratio (WHtR) and dual-energy X-ray absorptiometry (DXA) data were used.

BMI was calculated as the individual's weight in kilograms divided by the square of their height in meters (kg/m^2). In Sweden, at CHCs BMI charts include both standard deviation scores made by Karlberg et al.[27] and cut-offs for overweight and obesity according to IOTF [1], see Figure 2. For waist to height ratio, waist circumference in centimeters is divided by height in centimeters.

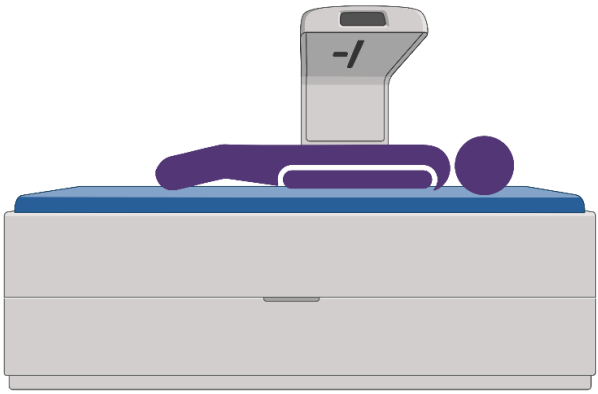

Figure 15, Illustration of a child lying in a DXA-machine, created in BioRender.com. Berggren, S. (2024) BioRender.com/h33o922

By the age of eight years, 281 children were invited for DXA assessment at Halland Hospital Halmstad. DXA scans were performed with the technician and the parent in the same room as the child, using the Lunar iDXA, ME+200663 (GE Healthcare Lunar, Madison, WI, USA). The scan settings were thin; OneScan 37 μGy, with the National Health and Nutrition Examination Survey (NHANES)/Lunar combined reference population matched by age and race. Fat mass index (FMI) was calculated by DXA-derived fat mass in kilograms divided by square height in meters, and presented in kg/m^2. Similarly, fat free mass index (FFMI) was calculated from DXA-derived total lean mass plus bone mineral content in kilograms, divided by the square height in meters and presented as kg/m^2. The sum of FMI and FFMI equals BMI. Total fat mass divided by total body mass equals fat percentage.

3.2.2 LABORATORY

For Study II-IV, non-fasting blood samples were used. Umbilical cord samples were drawn from the umbilical cord vein, containing mostly infant blood. Later blood samples were venous samples collected after local anaesthetics (EMLA (lidocaine/prilocaine), Astra Zeneca, Cambridgeshire, UK), almost exclusively during office hours (8 am-5 pm). Families were advised to turn in the blood sample as close to four months of age as possible, but a greater time span was

accepted the older the child became. In total, 449 samples were drawn at four months of age (range 107-148 days), 432 samples at 12 months (309-441 days) 359 samples at 36 months (1077-1173 days) and 328 samples at 60 months (1790-1884 days).

Samples were immediately frozen at -80˚C and stored until analysis. Total serum osteocalcin was analysed using the IDS-iSYS N-MID Osteocalcin assay technique (Immunodiagnostic Systems Limited, Tyne and Wear, UK). It is a chemiluminescence method of measuring osteocalcin amino acid 1-43 that equals to total serum osteocalcin but cannot not analyze the proportion of undercarboxylated osteocalcin. Results were expressed as µg/L. The Gothenburg Paediatric-Growth Research Center laboratory in Sweden were responsible for all analyses, which were made in duplicate. Samples from children born from 2008-2009 were analysed in the same assay batch in 2014. Remaining samples were analysed in 2022 in a second assay batch at the same laboratory using the same method. In 2022, the precision was evaluated using six serum controls, these were assayed using three lots of reagents in duplicate, twice per day, for 20 consecutive days on two systems. The lowest concentration was 5.9 µg/L, with a CV in full of 9.2%, and the highest was 149µg/L, with a CV in full of 4.8%.

3.2.3 NEURODEVELOPMENTAL ASSESSMENT

Close to the child's fourth birthday, at 48-51 months of age, parents picked a time for cognitive assessment at the pediatric clinic (Halland Hospital Halmstad). One trained child psychologist (BL) carried out all the cognitive assessments and the assessment of fine motor development for all children. These included the Swedish versions of the older age band (four to seven years) of the Wechler Preschool and Primary Scale of Intelligence, Third Edition (WPPSI-III) [202] and the manual dexterity area from the Movement Assessment Battery for Children, Second Edition (MABC-2)[203]. The data were transcribed by one nursing student (LH).

The WPPSI-III assesses verbal and cognitive performance in five different domains: full-scale IQ, verbal IQ, performance IQ, the processing speed quotient and the basal language quotient. Full-scale IQ is a composite score based on verbal IQ, performance IQ and processing speed aimed to provide an overall measure of a child's intellectual functioning. The score is standardized to a mean of 100.

MABC-2 is a practical test that includes posting coins, threading beads and a drawing trail test and evaluates motor skills and coordination. The test is

standardized and scored in an interval manner and designed to identify developmental difficulties.

Before the test situation, parents rated their child's cognitive and motor development at home using two screening tools, the Swedish version of the Ages and Stages Questionnaire, Third Edition (ASQ-III), a screening tool for developmental delays in children, and the Swedish version of the Strengths and Difficulties Questionnaire (SDQ), a child screening tool for emotional and behavioral problems.

ASQ-III [204] comprises a total score and five subdomains: communication, motor skills (gross and fine motor), problem-solving and social-emotional development. It is standardized on an interval scale and designed to identify potential developmental delays or areas of concern. The different age bands relate to specific age-related developmental milestones and the parent notices whether or not the child has reached the ability and or behavior yet.

The SDQ [205] comprises 25 questions that cover behavior in five different domains: emotional difficulties (anxiety and depression, internalizing emotional problems), conduct difficulties (externalizing behavioral problems like conduct disorder and aggression), hyperactivity difficulties (symptoms of attention deficit or hyperactivity), peer problems (social difficulties and peer-related issues) and a prosocial score (strengths such as empathy and cooperation). It is measured on a three-point ordinal scale that is not centered to the mean (0 = not true, 1 = somewhat true, 2 = certainly true). The total scores on each scale can be used separately to evaluate emotional and or behavioral problems.

3.3 STATISTICS

For Study I, on parental perceptions of childhood weight status, descriptive data were presented and the Chi-2-test performed to find potential differences between groups. A multiple regression analysis was outlined, to find sociodemographic factors that were associated to the parental ability to accurately perceive their child's weight status.

For Study II, blood samples from the reference population were used to compile reference limits for total serum osteocalcin. Scatterplots showed that osteocalcin was not normally distributed, except for the 4-months sample. Sex-specific reference limits for the following time-points, cord, 4, 12, 36, and 60

months were established by using medians and 97.5 and 2.5 percentiles, for boys and girls respectively. Spearman's non-parametric rank correlation test was used for covariate analysis. The covariates included child's sex, earlier osteocalcin levels, GA, size for GA, mode of delivery, single or twin birth and breastfeeding practices. Furthermore, maternal body mass index (BMI), diabetes, smoking and educational level were evaluated as possible covariates.

For Study III, associations between early osteocalcin levels and later anthropometrics and body composition were evaluated using Spearman's non-parametric rank correlation test.

For Study IV, linear regression analyses and spearman correlation analyses were performed for osteocalcin at the different ages and neurodevelopment at four years of age. A multivariable regression model was performed for osteocalcin at four months, full-scale intelligence quotient, sex, and parental educational level. Quartile groups based on the osteocalcin level were formed and children from the first and fourth osteocalcin quartile groups were then compared with regards to neurodevelopment. The Mann-Whitney U test was performed for group comparisons of nominal variables.

Significance was set at $p < 0.05$ for all included studies. The statistical analyses were performed using SPSS statistics for Windows, version 26.0 and 29.0 (IBM Corp, New York, USA) (see Paper I-IV for exact versions).

3.4 ETHICS

All procedures were carried out in accordance with the Declaration of Helsinki and approved by the Swedish Ethical Review Authority in Lund. For the first study of the 2,666 children enrolled in 2007-08 with anthropometrics and questionnaires, ethical permission was obtained (study no. 299/2007). The H^2GS Cohort with 396 included children in 2008-2009 and the follow-up with planned caesarean sections in 2010-2012 received study no. 44/2008. The Cord Clamping Study was also approved by the Swedish Ethical Review Authority in Lund (no. 41/2008, and follow-up no. 23/2012). For the study on osteocalcin and neurodevelopment, the ethical permissions were complemented with a request to merge research data (study no. 2018/940). Signed, informed consent was obtained from all the parents.

4 RESULTS

4.1 Parental perceptions of children's weight status (Paper I)

Study I examined whether parents of children with overweight or obesity perceived their children as weighing too much, and related these findings to sociodemographic factors, first at the age of two and then later, at the age of five. Of the 3,860 births during the recruitment period, 2,666 chose to participate in the study (69%), see Figure 14, method section. The inclusion criteria meant that somewhat fewer parents were born outside of Sweden, compared to the general population at the time. In total, 438 children (17.8%) had at least one parent born outside of Sweden, while both parents were born in Sweden for 2,016 children (212 missing replies), while for 144 children (5.4%), both parents were born outside of Sweden (227 missing replies). All continents were represented, with 75 different countries of birth. After five years, 1,862 (69.8%) of the initially recruited children were still enrolled in the study. The majority of households (61.5%) had at least one parent with university level education, (>12 years of schooling), 87.5% of children were born to non-smoking parents, see Paper 1 for details. At the five-year follow-up, there were no differences in sociodemographic factors between drop-outs and those who were still enrolled (parental age at childbirth, parental overweight, educational level, smoking habits or proportion of parents being born outside of Sweden ($p = 0.133–0.840$)).

In total, 14.9% of two-year olds and 11.8% of five-year olds were considered overweight or obese, according to BMI-SDS. (Table 1, Study I). When their parents were asked about their child's weight status, 96.4% at two years of age and 87.1% at five years of age answered that their child weighed just about right, Table 2.

The data show that parents increased their ability to identify overweight or obesity by the age of five, as compared to at two years of age ($p < 0.001$). The majority of parents, however, did not perceive their child's weight to be too much when the child's BMI was above cut-off levels for overweight at five years of age.

Table 2, Summary of parental weight perception by the child's actual weight category, in accordance with the body mass index-standard deviation score

	Number of parents who considered that their child weighed:			
	2 years		5 years	
Child's actual weight status	Just about right	Too much	Just about right	Too much
Normal weight	1,567 (99.9%)	1 (0.1%)	1,369 (99.7%)	4 (0.4%)
Overweight	295 (96.4%)	11 (3.6%)	182 (87.1%)	27 (12.9%)
Total (missing responses)	1,874 (792)		1,582 (1,084)	

The high number of missing responses is calculated from the point of enrolment and not for each year separately. The missing values were due to change of address, CHC or an active choice during these five years. The table is reproduced with permission from Acta Paediatrica

If parents were overweight themselves and/or had a low educational level, they had a higher probability of having a child with overweight or obesity ($p < 0.001$ and $p = 0.012$), but they were also more likely to misperceive their child's weight status, Table 4, Paper I. At five years of age, the odds ratio for misperception if the parent was overweight, as compared to normal weight, was 2.75 (95% confidence interval (CI) 1.80–4.21), and if the parent had low educational level as compared to a high educational level was 1.92 (CI 1.24–2.97). There were no differences in child weight status or parental ability to correctly classify their child's weight status based on child sex, parental nationality, smoking habits or maternal age, (Table 2, Study I).

Of the 209 children with overweight or obesity at 5 years of age, 171 children had complete anthropometrics and parental questionnaires for all four consecutive visits between two and five years of age. Of these, 54 children met the criteria for overweight or obesity at all visits. These 54 children had a higher BMI compared to the 117 children with overweight or obesity at the 5 year follow up who did not meet the criteria for overweight or obesity at all visits between two and five years of age, mean BMI 19.0 (SD 1.71) vs. BMI 18.0 (SD 0.77), (independent t-test, $p < 0.001$, mean diff 0.98 (95% CI 0.50 to 1.47). Of these 54 children, 12 parents (22.2%) perceived that their child weighed too much at five years of age, which may be compared to 10 parents of the 117 children (8.5%) with overweight or obesity at some but not all follow-ups between two and five years of age, (chi-2, $p < 0.05$).

4.2 REFERENCE LIMITS FOR OSTEOCALCIN (PAPER II)

For reference limit calculations, 459 of the 551 children were born full-term, appropriate for gestational age and to mothers without diabetes mellitus (see Method section). Of these children, 349 children were born in 2008-2009, of which 32 were born by caesarean section and 317 were born vaginally. Additionally, 110 children born by caesarean section in 2010-2012 were included in the reference population. Total serum osteocalcin differed with age and sex during the first five years of life. Levels in umbilical cord were low, but increased significantly for both sexes until four months of age; thereafter a decline was seen at one and three years of age, with rising levels at five years of age, see Figure 16. The pattern was similar for boys and girls but differed in exact limits. Boys had higher levels compared to girls at birth but lower levels from the age of one until five.

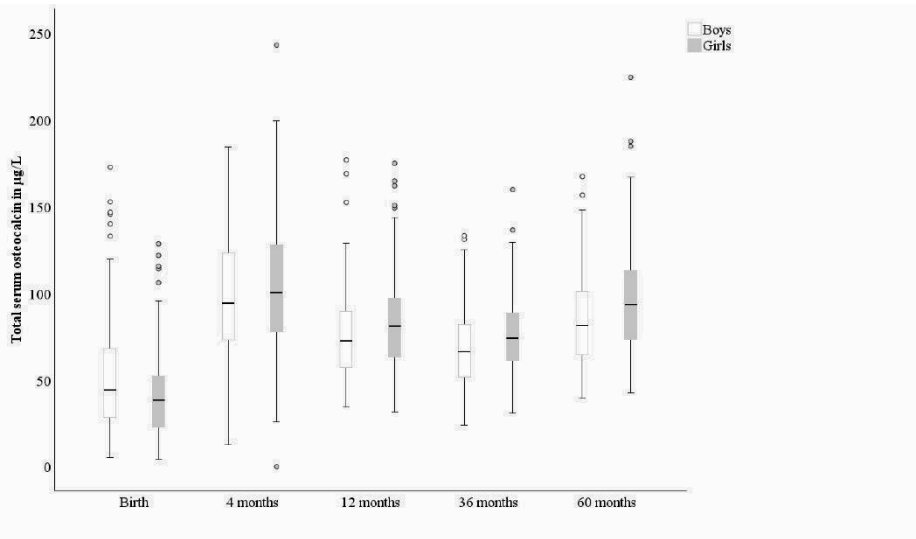

Figure 16, Boxplot showing total serum osteocalcin (µg/L), stratified by sex and measured by the IDS- iSYS N-MID osteocalcin assay technique, number of valid samples: Birth 431 (Boys 220), 4m 375 (Boys 193), 12m 359 (Boys 188), 36m 301 (Boys 158), 60m 274 (Boys 140)

In paper II, we discuss the timing for the infancy osteocalcin peak and we argue whether we missed the infancy osteocalcin peak for girls or whether girls do not present with the same peak as boys. Trying to visualize this, we made a boxplot, Figure 17, based on age in days at blood sampling for this PhD project. (Adding

gestational age did not change the overall picture). The majority of children turned in their blood sample between the age of 112-131days, therefore, the boxes on each end comprise few children, Figure 17. Visually, there seemed to be a peak in median osteocalcin around day 124-127 for boys, but the same visual pattern was not seen for girls.

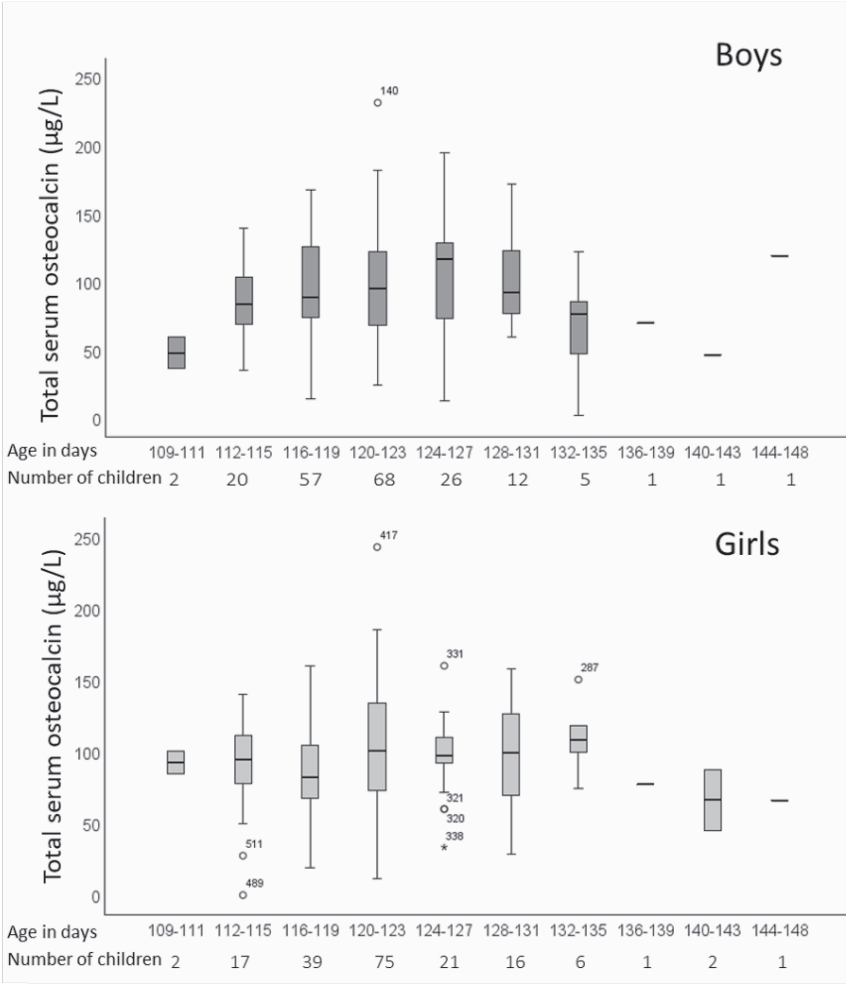

Figure 17, Boxplot showing total serum osteocalcin based on age in days at blood sampling for boys and girls, respectively (not adjusted for gestational age)

Umbilical cord osteocalcin showed a low correlation to later osteocalcin levels and was only significant for 12 months of age, Table 3, Study II. With increasing age, correlations to later osteocalcin levels got stronger, meaning that individual osteocalcin levels tended to vary less the older the child became.

For instance, osteocalcin at 12 months correlated to osteocalcin at 36 months, with a spearman Rho coefficient of 0.433, but from 36 to 60 months of age the Spearman Rho had increased to 0.626, Paper II, Table 3.

Table 3a Difference in median total serum osteocalcin at different time points and potential covariates

	Umbilical cord			4 months			12 months		
	median	n	*P**	median	n	*P**	median	n	*P**
Twin birth	49.1	17	**.040**	88.0	20	.884	62.6	17	**<.001**
Single birth	38.3	498		94.8	429		73.5	415	
Vaginally born	35.5	332	**<.001**	95.4	296	.666	72.5	282	.606
Born by Caesarean section	47.0	183		93.2	153		73.4	150	
Born SGA	32.5	29	.130	121.6	27	**.004**	74.7	25	.489
Born AGA	39.0	470		93.3	408		72.6	392	
Born LGA	35.1	16	.723	85.0	14	.885	73.4	15	.464
Born preterm	49.8	31	**<.001**	94.3	25	.839	73.3	28	.626
Born full term	38.3	453		95.1	398		72.6	381	
Born late term	33.2	31	**.016**	84.8	26	.245	76.4	23	.256
Only breastfed at 4 months				100.7	249	.653	73.9	243	.160
Partly breastfed at 4 months				104.5	107		70.1	103	
Only formula fed at 4 months**				73.8	89	**<.001**	72.1	83	.650
Partly breastfed at 12 months							101.8	35	**.049**
Not breastfed at 12 months/No human milk							72.2	336	
Maternal diabetes	23.1	18	.074	95.9	18	.493	85.5	18	.419
No maternal diabetes	39.3	394		95.4	364		73.1	352	
Mother with BMI <25	40.5	284	.230	94.4	265	.287	73.1	250	.536
Mother with BMI ≥25	37.1	123		97.5	111		73.8	114	

Table 3b Difference in median total serum osteocalcin at different time points

	36 months			60 months		
	median	n	*P**	median	n	*P**
Twin birth	62.6	16	.177	79.8	15	.258
Single birth	69.0	343		88.2	313	
Vaginally born	65.8	235	**<.001**	85.2	212	.471
Born by caesarean section	75.3	124		90.6	116	
Born SGA	75.5	24	.092	92.0	24	.355
Born AGA	67.6	322		85.4	295	
Born LGA	76.6	13	.156	107.3	9	.107
Born preterm	82.1	23	**.006**	86.1	23	.645
Born full term	67.0	319		85.4	292	
Born late term	82.7	17	**.046**	100.7	13	.147
Only breastfed at 4 months	65.7	204	**.016**	83.0	188	**.043**
Partly breastfed at 4 months	71.8	89		93.7	81	
Only formula fed at 4 months**	72.9	64	.603	92.0	58	.828
Partly breastfed at 12 months	69.5	27	.517	97.9	26	.163
Not breastfed at 12 months / No human milk	68.1	285		86.2	259	
Maternal diabetes	74.7	16	.298	106.6	15	.340
No maternal diabetes	68.1	299		86.6	273	
Mother with BMI <25	66.9	211	**.027**	84.9	191	.216
Mother with BMI ≥25	73.0	98		92.7	91	

*P = P value, calculated with the Mann-Whitney U test. Bold type indicates $P < 0.05$.
**p-values for only breastfed at 4 months compared to only formula fed at 4 months were < 0.001 at 4 months of age; 0.451 at 12 months, 0.271 at 36 months and 0.060 at 60 months. SGA, small for gestational age; AGA, appropriate for gestational age; LGA, large for gestational age, BMI = Body mass index, n = number of children

At four months of age, osteocalcin was higher for breastfed children (exclusively and partially breastfeed) compared to only formula, Table 3a, (Spearman rho -0.308) and for children born small for gestational age (Spearman rho -0.117). At 12 months twins showed lower median osteocalcin compared to single born children (Spearman rho -0.177), but this did not last until three years of age. At three years of age, vaginally born children showed higher osteocalcin levels compared to children born by c-section, Table 3b (Spearman rho 0.190). Children born pre-term (Spearman rho 0.148) and children born post-term (Spearman rho 0.109) showed higher median osteocalcin levels at three years of age compared to children born full-term, Table 3b. Children born to mothers with overweight had higher osteocalcin levels at three years of age compared to children born to mothers with normal BMI at gestation, Table 3b, (Spearman rho 0.135). Furthermore, children that received formula (with or without complementary breastfeeding) at four months of age showed higher osteocalcin levels at three and five years of age, compared to children who were exclusively breastfed at four months of age, Table 3b (spearman rho 0.141 resp. 0.124).

4.3 OSTEOCALCIN IN RELATION TO GROWTH AND BODY COMPOSITION (PAPER III)

For boys, umbilical cord osteocalcin correlated with height and weight until four months of age, but not to later measurements. No correlations were found for boys' umbilical cord osteocalcin and waist circumference, WHtR or BMI until eight years of age. No correlations were found for girls' umbilical cord osteocalcin and height, weight, waist circumference, WHtR or BMI at any time points until eight years of age.

In infancy, the 4-month osteocalcin sample correlated in a negative way to weight and waist circumference at all time points until two years of age for boys, but only to weight at four months of age and waist circumference at 4, 6, and 18 months for girls, see Table 4. Boys' 1-year osteocalcin correlated in a negative way to waist circumference and WHtR at 2 years of age (Spearman rho -0.204, $p = 0.006$, $n = 181$) but in a positive way for girls (Spearman rho -0.177, $p = 0.028$, $n = 153$). Furthermore, girl's osteocalcin at one year of age correlated to all later weight measurements, and to later WHtR and BMI in a scattered way, this while no correlations were seen for boys' osteocalcin at one year and later anthropometrics (except for waist circumference and WHtR at two years of age, Table 2-5, Paper III). From three years of age, osteocalcin

correlated to all later height and weight measurements for girls until eight years of age, Table 5. For boys, correlations were found for osteocalcin at three and five years of age and later height and weight until six and a half years of age but not at eight years of age, Table 5. Boys' osteocalcin at three and five years of age did not correlate to waist circumference, WHtR or BMI. For girls, osteocalcin at three and five years of age correlated to waist circumference, WHtR and BMI in a scattered way, Table 5.

Table 4, Total serum osteocalcin at four months of age in relation to later anthropometric measurements for boys and girls respectively

Boys' osteocalcin at 4 months of age (µg/L)		4 M	12 M	18 M	2 Y
		Height (cm)			
	ρ	-.116	-.131	-.105	**-.181***
	P	.077	.050	.130	**.009***
	n	232	225	210	209
		Weight (Kg)			
	ρ	**-.136**	**-.163**	**-.156**	**-.149**
	P	**.038**	**.014**	**.023**	**.030**
	n	232	225	211	213
		Waist circumference (cm)			
	ρ	-.124	**-.203***	**-.155**	**-.185**
	P	.078	**.004***	**.033**	**.013**
	n	204	196	190	182
		Waist to height ratio (WHtR)			
	ρ	-.049	**-.146**	-.119	-.078
	P	.484	**.041**	.102	.301
	n	204	196	190	180
		Body mass index (BMI)			
	ρ	**-.146**	**-.154**	-.036	**-.143**
	P	**.026**	**.021**	.602	**.038**
	n	232	225	209	210

Girls' osteocalcin at 4 months of age (µg/L)		4 M	12 M	18 M	2 Y
		Height (cm)			
	ρ	**-.229***	-.083	-.031	.028
	P	**.001***	.230	.672	.703
	n	217	210	191	182
		Weight (Kg)			
	ρ	**-.161**	-.070	-.038	-.060
	P	**.017**	.311	.597	.422
	n	217	210	191	183
		Waist circumference (cm)			
	ρ	**-.177**	-.117	**-.158**	-.076
	P	**.018**	.118	**.048**	.350
	n	178	179	157	154
		Waist to height ratio (WHtR)			
	ρ	-.040	-.115	**-.170**	-.086
	P	.592	.126	**.033**	.292
	n	178	179	157	154
		Body mass index (BMI)			
	ρ	-.061	-.028	-.066	-.031
	P	.371	.688	.378	.666
	n	217	210	182	191

M, months of age; Y, years of age; ρ, Spearman's rho; P, significance level; n, number of children in calculation. * Correlation was significant at the 0.01 level (2-tailed). Bold type indicates that correlations were significant at the 0.05 level (2-tailed).

Table 5, Total serum osteocalcin at three and five years of age for boys and girls respectively and later anthropometric measurements

Boys' osteocalcin at 3 years of age (µg/L)		3 Y	5 Y	6.5 Y	8 Y
		Height (cm)			
	ρ	.137	**.172**	**.187**	.099
	P	.061	**.020**	**.025**	.268
	n	188	184	144	128
		Weight (Kg)			
	ρ	.113	**.149**	.104	.027
	P	.122	**.043**	.214	.761
	n	188	184	144	128

Boys' osteocalcin at 5 years of age (µg/L)	5 Y	6.5 Y	8 Y
	Height (cm)		
ρ	**.214***	**.166**	.145
P	**.006***	**.050**	.110
n	166	140	123
	Weight (Kg)		
ρ	**.234***	**.171**	.152
P	**.002***	**.043**	.093
n	166	140	123

For boys' osteocalcin levels at 3 and 5 years of age, no correlations were found for waist circumference, waist to height ratio (WHtR) or body mass index (BMI)

Girls' osteocalcin at 3 years of age (µg/L)		3 Y	5 Y	6.5 Y	8 Y
		Height (cm)			
	ρ	**.178**	**.186**	**.241***	**.207**
	P	**.020**	**.016**	**.005***	**.022**
	n	170	166	133	123
		Weight (Kg)			
	ρ	**.174**	**.195**	**.260***	**.243***
	P	**.023**	**.012**	**.003***	**.007***
	n	170	166	133	123
		Waist circumference (cm)			
	ρ	.102	.085	**.218**	.178
	P	.189	.288	**.012**	.058
	n	168	160	132	114
		Waist to height ratio (WHtR)			
	ρ	.029	.036	.149	.137
	P	.705	.652	.097	.147
	n	168	160	126	113
		Body mass index (BMI)			
	ρ	.146	.143	**.222**	**.199**
	P	.058	.066	**.012**	**.035**
	n	170	166	127	113

Girls' osteocalcin at 5 years of age (µg/L)	5 Y	6.5 Y	8 Y
	Height (cm)		
ρ	**.184**	**.236***	**.258***
P	**.019**	**.006***	**.003***
n	162	135	126
	Weight (Kg)		
ρ	**.168**	**.255***	**.250***
P	**.032**	**.003***	**.005***
n	162	135	126
	Waist circumference (cm)		
ρ	.142	**.273***	**.209**
P	.071	**.001***	**.024**
n	162	134	117
	Waist to height ratio (WHtR)		
ρ	.108	**.223**	.125
P	.170	**.011**	.180
n	162	129	116
	Body mass index (BMI)		
ρ	.149	**.223**	.181
P	.058	**.011**	.052
n	162	130	116

M, months of age; Y, years of age; ρ, Spearman's rho; P, significance level; n, number of children in calculation. *Correlation was significant at the 0.01 level (2-tailed). Bold type indicates that correlations were significant at the 0.05 level (2-tailed).

Osteocalcin levels at three years of age for girls and at five years for boys showed correlations to total body mass and bone mineral content assessed with DXA-data at eight years of age, Table 6. Correlations were also found for girls' osteocalcin levels at three and five years of age and for total body fat and fat mass index but not to fat-free mass index. Boys showed the opposite pattern, with correlations between the five-year osteocalcin sample and total lean mass and fat-free mass index but not for fat mass index or total body fat, Table 6.

Table 6, Total serum osteocalcin in μg/L at different time points, in relation to DXA scans at eight years of age and stratified by sex.

		Dual energy x-ray absorptiometry at 8Y							
		Total tissue %	Total body mass	Total body fat	Total Lean mass	Bone mineral content (g)	FMI	Fat %	FFMI
		Boys							
3 Y	ρ	-.100	.009	-.060	.078	.083	-.095	-.097	.026
	P	.299	.927	.534	.417	.387	.323	.311	.785
	n	111	111	111	111	111	111	111	111
5 Y	ρ	.019	**.209**	.104	**.269***	**.239**	.063	.017	**.212**
	P	.840	**.028**	.281	**.005***	**.012**	.515	.858	**.026**
	n	110	110	110	110	110	110	110	110
		Girls							
3 Y	ρ	**.197**	**.223**	**.224**	**.215**	**.227**	**.222**	**.196**	.146
	P	**.039**	**.019**	**.019**	**.024**	**.017**	**.020**	**.040**	.128
	n	110	110	110	110	110	110	110	110
5 Y	ρ	.173	**.200**	**.211**	**.202**	.168	**.194**	.175	.078
	P	.066	**.034**	**.025**	**.032**	.075	**.039**	.063	.412
	n	113	113	113	113	113	113	113	113

Y, years of age; FMI, Fat mass index; FFMI, Fat-free mass index; ρ, Spearman's rho; P, significance level; n, number of children in calculation. * Correlation was significant at the 0.01 level (2-tailed). Bold type indicates that correlations were significant at the 0.05 level (2-tailed).

4.4 OSTEOCALCIN IN RELATION TO NEURODEVELOPMENT (PAPER IV)

In our final paper of this PhD project, about osteocalcin and neurodevelopment, the sample consisted of 158 vaginally born, term-born children. There was an association between osteocalcin at four months of age and a higher full-scale intelligence quotient at four years of age (r^2 0.031, ß 0.049, $p < 0.05$, Table 7). Regression analyses were significant for osteocalcin levels at 4 months of age and Performance IQ (WPPSI-III), Drawing trail test (MABC-2) and the gross motor subdomain (ASQ-III), Table 7.

Table 7. Significant linear regression analyses between total serum osteocalcin at four months of age and neurodevelopmental assessment at four years of age (unadjusted) [a]

	Age at blood test	N	Intercept	R^2	ß	95% CI	P	ρ
Full-scale IQ (WPPSI-III)	4M	139	112.1	.031	.049	0.003-0.095	**<.05**	**.171**
Performance IQ (WPPSI-III)	4M	138	110.8	.036	.041	0.005-0.077	**<.05**	.145
Drawing trail test (MABC-2)	4M	137	8.0	.054	.012	0.004-0.060	**<.05**	**.266**
Gross motor (ASQ-III)	4M	141	52.5	.029	.034	0.001-0.066	**<.05**	.102

[a] Neurodevelopmental assessment included: WPPSI-III (full-scale IQ, verbal IQ, performance IQ, processing speed quotient, basal language quotient). ASQ-III (Total score and five subdomains: communication, motor skills (gross and fine motor), problem-solving and social-emotional development. MABC-2, (manual dexterity score, posting coins, threading beads, drawing trail test). Non-significant regression analyses were not reported. Bold type indicates significant correlations ($p<0.05$). R^2, R square; ß, Unstandardised ß coefficient; P, significance level; ρ, Spearman's rho; The table is reproduced with permission from Acta Paediatrica.

Children with osteocalcin levels in the highest quartile scored 5.6 (95% CI 1.3-9.9, n = 34) points higher for full-scale IQ than those in the lowest quartile (n=35), mean scores 118.8 ± 8.8 and 113.2 ± 9.2 ($p < 0.05$). For performance IQ the numbers were, mean 116.2 (SD 7.0) for the highest quartile, with a mean difference of 4.1 (CI 0.6-7.6) to the lowest quartile, $p < 0.05$. Children within

the highest osteocalcin quartile also scored higher on the ASQ-III, gross motor subtest (mean 57.6 (SD 3.5), mean difference 4.3 (95% CI 0.7-7.9), n = 36, $p < 0.05$) and scored higher in the MABC-2 drawing trail test (mean 9.8 (SD 1.5) mean difference 1.1 (95% CI 0.4-1.9), n = 34, $p < 0.05$), compared to children within the lowest osteocalcin quartile (n = 36 resp. 35). Cord levels of osteocalcin were negatively associated with processing speed and fine motor development at four years, Table 1, Paper IV. Levels at 12 and 36 months of age were not associated with neurodevelopment at four years of age.

The SDQ screening, measuring social or behavioral problems, was completed by parents of 155 children. For the total difficulties score, 145 children had normal screening results, eight children screened borderline and two children had abnormal scores; there were similar numbers for included subdomains. There were no differences in SDQ scores between the quartile groups, but power was low.

A multivariable regression model for osteocalcin at 4 months of age, full-scale IQ at 4 years, sex and parental education was created, Table 2, Paper IV. Osteocalcin remained a significant contributor to full-scale IQ in the model and showed an unstandardised coefficient of 0.05 for osteocalcin levels at 4 months of age. According to Study II, median osteocalcin levels at 4 months of age were 94.4 μg/L (2.5 percentile: 21.6 μg/L, 97.5 percentile: 170.5 μg/L). The model showed that female sex, added a mean of 5.0 points compared to boys as a control, and having highly educated parents, adding a mean of 5.7 points on full-scale IQ, Table 2, Paper IV.

5 DISCUSSION

One aim of these studies was to explore parental perceptions of childhood overweight and obesity, and to increase knowledge about infant and childhood osteocalcin, in order to better understand potential barriers in weight management. Another aim was to increase knowledge on childhood osteocalcin in relation to cognition and brain development. Our results showed that hardly any parents perceived that their child weighed too much, even when BMI charts showed that the child had overweight or obesity by the ages of two or five. Parents who were overweight themselves or had a low educational level were more likely to misperceive their child's weight status. However, because of the overall low agreement between perceived weight status and actual weight status parental weight status or educational level would be of minor clinical importance. In our second study, we presented age- and sex-specific reference limits for osteocalcin from birth until five years of age, and related these levels to early environmental factors. Osteocalcin was higher if the child was born at earlier gestational age, and breastfeeding correlated to higher osteocalcin-levels while it was ongoing. Thereafter, we found correlations between early osteocalcin levels and later growth and body composition, which were negative during infancy but positive during childhood. Osteocalcin during childhood correlated to fat-free mass index for boys but to fat mass index for girls, indicating that osteocalcin might have different roles based on sex. Lastly, we show that osteocalcin at four months of age was positively correlated to intelligence quotient and motor development at four years of age.

5.1 PARENTAL PERCEPTIONS OF CHILDREN'S WEIGHT STATUS, STUDY I

In our Swedish cohort, only 4% of parents of two-year-old children and 13% of parents of five-year old children with overweight or obesity replied that their child weighed too much. Parents that were overweight themselves or had a lower educational level were even less likely to acknowledge that their child weighed too much, compared to parents with normal weight or a high educational level. Our numbers showed lower or similar agreement between actual and perceived weight status as seen in other parts of the world [80-82]. Educational level is, however, one factor that consistently associates to a higher level of agreement between perceived and actual weight status [84, 87,

206]. In our cohort, 61.5% had at least one parent with 12 or more years of school, equivalent to university level, which may be considered rather high. Still, the agreement between perceived weight status and actual weight status was low in our cohort, indicating that it is not meaningful to pinpoint specific groups for interventions, since the vast majority would need similar interventions. In a meta-analysis of children 0-19 years of age [206], factors associated with misperception were younger children, male sex, higher child BMI and ethnicity, along with higher parental BMI and lower educational level. There is evidence that ethnicity may be of importance, even though we did not find support for this, and our study showed that parents born outside of Sweden were somewhat underrepresented. One explanation as to why ethnicity may be of importance could be that cultures with higher prevalence of obesity may also show higher acceptance, because it has been normalized [207-210]. If normalization would explain the low agreement between actual and perceived weight status seen in our study, then prevalence numbers for obesity should also be among the higher in Sweden, but that is not the case [64]. This is why normalization cannot explain the low agreement between actual and perceived weight status among Swedish parents. However, our study included children of younger age than most of the other studies listed above, and this may be the reason for the somewhat higher degree of misperception seen in our study, compared to others as shown in the meta-analysis by Alshahrani et al. [206].

A limitation of this study was, however, the wording of the question: “Do you think that your child weighs: Too much, just about right, or too little?”. This question did not specify that “too much” was intended to relate to weight for height or having a high BMI. Parents could then have interpreted the question in such a way that they thought that their child’s weight was “just about right”, taking the standpoint that their child was perfect, with or without overweight not to judge or criticize their child. Furthermore, psychological aspects like idealization is common among parents [96], meaning that parents tend to idealize their child to have a normal weight. If the question had been stated “high BMI” or “weight for height”, that might have resulted in responses that were different than those we received. Furthermore, it is likely that this question is influenced by a social desirable bias, as discussed by Rodrigues et al., who published one of the more recent articles on the topic [211]. Rodrigues et al. also proposed that social desirable bias, might be one reason to why no improvement in parental ability to correctly estimate child’s weight status has been seen over the years, despite increasing societal knowledge about health risks associated with childhood obesity.

For parents of children with overweight or obesity at all time points from two until five years of age, 22% perceived that their child weighed too much. This is somewhat better than what was seen for all children with overweight and obesity at five years of age (13%). Still, the majority of parents did not think that their child weighed too much, despite four check-ups showing that the child had overweight or obesity. This means that parents either turn a blind eye to the problem or that professionals at the BVC do not acknowledge the problem at the visits. Research shows that nurses at CHCs experience childhood obesity to be a difficult topic, associated with negative feelings for both parents and health professionals, and that communication about childhood obesity could compromise the medical relationship [101, 212]. Therefore, nurses tend to avoid the subject, so as not to hinder future contacts, which could also hinder future vaccinations and other interventions [98]. Similarly, another study found that 24% of parents would, in fact, avoid future medical appointments in response to perceived weight stigmatization by providers, and 33% would change their doctor in response to feelings of weight stigmatization [213]. Perhaps, communication difficulties and weight stigmatization are some of the keys to the low proportion of parents perceiving that their child weighed too much, even after three years and four visits to the CHC showing that the child had overweight or obesity. But, if parents do not identify overweight or are not aware of childhood overweight as a health issue, the child is left alone without help. Therefore, health professionals working at the CHC need to be aware that obesity is associated with weight stigma [99], but still address the subject. It is important to approach the subject with respect and in a humble way, given that discussing childhood obesity can evoke feelings of inadequacy in parents [100]. The approach is important to ensure future follow-ups and prevent the child from having an unnoticed and treatable disease. Although, the best approach to do so remains unclear [100], one way to assess body composition is by using growth charts as a tool. Growth charts might help to raise awareness – for both professionals and parents – and may be a good way to start the discussion.

This study was conducted on children born 2007-08. Since then, the media has picked up the topic of increased prevalence of obesity on all levels in society. Despite media coverage, newer studies do not imply a better agreement between perceived and actual weight status among children with overweight or obesity [211]. Nevertheless, I still hope and believe that general knowledge of health-related issues regarding childhood obesity, is higher today compared to when the parents in Study I replied to the questionnaires. Nowadays, when I work at the CHC, it happens that parents ask me about their child's weight

status according to BMI, or ask for a discussion on the topic. This is something that I never experienced when I started working at the CHC in 2017. Perhaps, I am more responsive to those contacts because of my interest in the question, but I do believe that, although overall awareness is still low, it has increased since the parents in Study I replied to the questionnaires. This experience is supported by a study performed by Ruiter et al. [214]. Ruiter et al. investigated the difference in parental perception of childhood weight status and actual weight status, between 2009 and 2013, in the Netherlands, and showed that underestimation decreased over the years, but was still unsatisfactorily low. Yet, even with increased societal awareness, there is a risk that underestimation continues, linked to normalization of overweight and obesity, as the prevalence of the conditions continues to increase [209]. Furthermore, overweight is an increasing problem also for parents, and parental BMI is one factor that repeatedly has been associated with misperception of childhood overweight [206]. Furthermore, stigma does not seem to lessen, which is why parents might "protect" their children, by ignoring the issue, against their better judgement, or continue to underestimate their children's weight status based on social desirable bias [99, 211, 213].

5.2 REFERENCE LIMITS FOR OSTEOCALCIN, STUDY II

Little is known about osteocalcin in infancy and early childhood. The second study in this thesis adds to the knowledge, by presenting reference limits during the first years of life for total serum osteocalcin. Previous studies have often used broad age intervals or non-sex stratified data. Our results show that osteocalcin follows a wave-like pattern, with a peak somewhere around four months of age. The existence of an infant peak has earlier been questioned [137, 141]. We suggest that the reason for these conflicting results are wide age groups and a relatively high variability between samples, which, together with relatively few samples, would easily hide a true peak. Furthermore, we show that osteocalcin presents with sex differences from the age of one.

To understand the natural pattern of osteocalcin and the variation in osteocalcin levels seen between individuals, we continued to investigate early factors and their possible association to later osteocalcin. One of these factors was gestational age. The earlier a child was born, the higher was the umbilical cord osteocalcin. Although differences diminished by the age of four months, at three years of age, children born preterm or post-term still had higher

osteocalcin levels, compared to children born full-term. Additionally, feeding practices seem to associate to osteocalcin levels, at least at time, since breastfed children had higher levels compared to formula-fed at four and 12 months of age. However, we also found that, years after breastfeeding practices had ended, children that had been exclusively breastfed at four months of age had lower osteocalcin levels at three years of age. With regard to early programming, early-life feeding practices are likely to be of importance. For instance, lactating mice receiving milk from over-nurtured dams showed a larger leptin surge and become fatter during the suckling period [102]. But, if the pup received regular chow after day 23, they still presented with leptin resistance in the arcuate nucleus, despite normal leptin levels [103]. Additionally, these pups rapidly developed obesity and insulin resistance in response to a high-fat diet later in life, as compared to littermates that had been suckling from dams receiving regular chow [103]. At present, not enough is known about osteocalcin and its potential functions, but pathways between leptin and osteocalcin are close, and the result presented by Glavas et al. [103] may be one clue as to why some individuals are more susceptible to obesity than others. This is why we find it interesting that early feeding practices associate with osteocalcin levels at the time and later – a topic that is further discussed in our third and fourth studies. Another interesting finding of our second study was that the older the child became, the stronger the correlation was for osteocalcin between two adjacent time points. So, with age, early osteocalcin levels tend to become a stronger predictor of later osteocalcin levels, even with similar variation on population level.

One unexpected finding that we decided to omit in Paper II, and that has not previously been published in literature, was that mode of delivery showed correlations to later osteocalcin levels. This computation was only performed because the study design had originally included children mostly born vaginally in 2008-2010, and then with caesarean section in 2010-2012. We wanted to make sure that mode of delivery was *not* a factor that would impact osteocalcin levels. However, the calculations showed that children born vaginally showed lower osteocalcin levels, not only at birth but also at three years of age. Adjusting for gestational age and twin births did not change this, but rather showed that also being a twin meant higher osteocalcin levels at birth and at 12 months of age. These results should be interpreted with care, given that there is a risk for mass significance, and no previous theory would explain these results. Nevertheless, the findings stood for adjustment. When publishing the related paper, we chose to omit these results, on the basis of feedback that the manuscript was too complicated and difficult to read. Still, we believe that

the information, that mode of delivery or being born as a twin correlated to later osteocalcin levels, builds on the knowledge, which is why we have chosen to publish these data here, even with a lack of interpretation. Perhaps, these results will constitute a missing piece of someone else's puzzle in the future.

5.3 OSTEOCALCIN AND GROWTH, STUDY III

Osteocalcin probably has several different actions in the body, as described in the Introduction to this thesis. In the battle to reduce childhood obesity, osteocalcin might be one important player, because it shares pathways with leptin and adiponectin, increases insulin sensitivity and is associated to lower BMI among adults [155, 156, 164, 190].

In our third paper, we show that osteocalcin levels in infancy correlated in a negative way to growth until two years of age. Moreover, osteocalcin levels at three years for girls and five years for boys correlated in a positive way to height, weight, and waist circumference until six and a half to eight years of age. Osteocalcin levels at three years for girls correlated to fat mass index and total body fat at eight years of age while osteocalcin levels at five years of age for boys correlated to fat-free mass index and total lean mass at eight years of age. This might be the first study to investigate early osteocalcin levels in relation to later anthropometrics and later body composition assessed with DXA. Our results confirm that early osteocalcin levels carry information about later growth and body composition.

The study design, however, does not answer the question as to why correlations during infancy were negative, while correlations in childhood were positive. One reason could be that children with high levels of osteocalcin at four months of age had a more intense growth spurt during infancy, because they were smaller from the beginning. If so, osteocalcin could either act as a mediator or mirror of this rapid growth, rather than correlating to size at time. Another similar explanation could be that children with high osteocalcin levels at 4 months have a more rapid growth pattern (as illustrated in Figure 3, method section), and thereby have a lower BMI at 1 year, compared to children with a slower growth rate. Another reason for this shift from negative to positive correlations could be different actions during different periods of life. Perhaps, this inverse relationship during infancy is connected with the coinciding period known as "mini-puberty". During this phase, normally occurring around 2 months of age, estrogen levels are low compared to pubertal levels, but transiently higher than at earlier and later ages [215]. This theory is supported

by the fact that previous research found inverse relationships between osteocalcin levels and BMI from the onset of puberty in adolescence until adulthood [155-157]. Furthermore, estrogen levels and osteocalcin levels are dependent on each other, and osteocalcin levels decrease when women start hormonal replacement therapy or contraceptives [216].

At five years of age, osteocalcin correlated to total body mass for both sexes, but the finding that I find the most interesting was that girls' osteocalcin correlated with fat mass and boys' osteocalcin to lean mass. The finding was unexpected, and correlations are weak. Nevertheless, osteocalcin levels measured as early as three years for girls and five years for boys predicted about 4-5% of the variation seen in total body fat for girls and about 7% of total lean mass for boys at eight years of age. This supports the possibility that osteocalcin may have a sex-specific programming potential. This may potentially explain why previous studies have found conflicting results when it comes to lean and fat mass in mixed sex populations [159]. In an experiment with mice, Yasutake et al. [217] showed that if osteocalcin was administered orally over a long term in female mice given a high fat, high sucrose diet, glucose tolerance increased while fasting blood glucose levels decreased. However, when similar experiments were performed on male mice, insulin resistance, glucose intolerance and adipocyte hypertrophy were seen [218]. This supports the possibility that osteocalcin might have different effects in male and female subjects, even when this study showed correlations for fat mass in males and not girls, contrary to our findings. These sex-differences were further elucidated in an experiment with orchiectomized male mice, which showed similar results as for female mice, while the opposite phenotype was seen for female mice given testosterone [218]. This suggests that there might be sex-specific action of the bone–energy homoeostasis axis, perhaps via adiponectin in females and testosterone in males [219]. In the study by Wang et al. [159] on children aged 7-12 with obesity, osteocalcin showed positive correlations with lean body mass, but inverse correlations with fat percentage. This population differed from ours, given that children had obesity, were older and that it was a mixed sex population with boys making up 55 out of the 79 children. When it comes to fat mass correlations, we found positive correlations for girls and no correlations for boys, while Wang et al. found negative correlations for their mixed sex population [159]. Perhaps fat mass and osteocalcin correlates even for boys when fat mass reaches abnormal proportions. Or, because body composition differs between the sexes during all stages of growth, from infancy to puberty [220-222], perhaps the mixed-sex design with 24 participants being girls, are the reason for their correlation

[159]. Another reason for negative correlations to fat mass were the older age in their population, given that it is likely that correlations turn negative before the onset of puberty [155-157].

Osteocalcin is known both as a bone formation marker and a metabolic active hormone. Therefore, it is interesting that our study showed the strongest and most consistent correlations towards weight and waist circumference and not towards height or height-adjusted markers of body composition, such as WHtR or BMI. Similarly, for osteocalcin at five years of age, girls' osteocalcin correlated to total body mass but not to bone mineral content, indicating that osteocalcin is more than a bone formation marker. Although, assessment of bone mineralization with DXA data are fraught with difficulties in the interpretation [223], these results are still interesting. Perhaps the hormone osteocalcin should be thought of in a similar way as IGF-1, as opposed to being thought of as a bone-formation marker. IGF-1 is an anabolic hormone with importance for growth and has a close relationship to osteocalcin [224]. Giving a mouse an anti-IGF1 antibody decreases osteocalcin activity in osteoblasts. IGF-1 and insulin both interact with FOXO1, which regulates the expression of the mouse osteocalcin gene (Bglap2) [225]. While both IGF-1 and osteocalcin promote bone growth, IGF-1 also stimulates the differentiation of preadipocytes and increases muscle mass, and it is possible that osteocalcin has similar effects [24]. Another similarity between the hormones, indicating a relationship between the hormones, and a relationship to nutrition and growth, was that IGF-1 was significantly different for formula-fed children [226], just like what we showed for osteocalcin.

In the lab by Karsenty, Mera et al.[227] showed that OC-null mice had reduced muscle mass and smaller muscle fibres compared to wild type mice, but the results could not be reproduced by Moriishi et al. [117], which showed no effect on OC-null mice on muscle mass. Whether or not there is an association between osteocalcin and muscle mass, the association to fat mass has been somewhat more studied. Kindblom et al. [228] showed that fat mass was inversely associated to osteocalcin in humans and, in a meta-analysis by Liu et al. [155], the association to BMI and percentage body fat was concluded for adults. Tubic et al. [229] did, however, argue that osteocalcin does not correlate to BMI, but, rather, to rapid weight gain, and they showed that, for girls with anorexia nervosa who rapidly gained fat mass, osteocalcin increased. This is especially interesting, given that, in our study, osteocalcin at 5 years of age correlated to fat mass for girls but not boys, measured by DXA at eight years of age. BMI is dependent on fat mass and lean mass, and differs with age and

sex. The absent association between BMI and osteocalcin in the study by Tubic et al. [229] may therefore be explained by the fact that they only included girls who, additionally, had an altered body composition with a very low fat mass due to their condition. Our results suggest that osteocalcin is related to lean mass for boys but to fat mass for girls at eight years of age, which both impact total body mass and BMI. Still, our longitudinal data shows that osteocalcin at three and five years, respectively, were associated to body mass at later years, which supports the possibility that osteocalcin is related to weight gain for both sexes, but only to fat mass for girls.

When discussing factors that correlate to early osteocalcin levels, it is important to mention that our study design does not say anything about causality or give explanations for our findings. One theoretical explanation for our findings could be that lower osteocalcin at three years of age is a sign of a later adiposity rebound, rather than a low amplitude. If early feeding practices impacts osteocalcin levels at the time and later in life, and if osteocalcin levels in turn impact the timing of the infancy growth peak, and perhaps also the adiposity rebound, these found correlations could be the signal that links early feeding practices to later growth. Osteocalcin has been shown to increase during rapid weight gain [229], which could explain the rise in osteocalcin at four months and five years, but could also explain why children born SGA present with the highest osteocalcin levels of all at four months of age, given that SGA children often present with rapid growth during the first months of life. The negative correlations between infant osteocalcin and growth in infancy could therefore be signs of growth tempo or pattern, rather than a correlation to prevalent height or weight. This theory would also explain the higher levels seen for children born pre- or post-term, in that children born early or late both have an increased risk for an early adiposity rebound. These results strengthen the suggestion that osteocalcin might be involved in early programming, either as a mirror or a mediator. This is also why early osteocalcin levels and factors that impact these levels might be of importance for later health and why the correlation to early environmental factors in Study II are interesting.

5.4 OSTEOCALCIN AND NEURODEVELOPMENT, STUDY IV

In the fourth study, we show that high osteocalcin at four months of age correlates to higher full-scale IQ, performance IQ and to a more mature motor

control at four years of age. Full-scale IQ is a composite score that assesses both spatial visualization and short- and long-term memory function, which are hippocampal functions. Our study design shows statistical correlations and not causality, but given that osteocalcin is known to bind to the GPR158 receptor in the hippocampus, where it stimulates the histone-binding protein RbAp48 [174, 175], there is a theoretical framework for plausibility. A decrease in RbAp48 expression is a molecular indicator of memory loss that correlates with aging in humans and mice [175]. There is also an analogy to the experiments performed by Khrimian et al., who reversed age-related cognitive decline in mice by injecting them with plasma from young wild type mice. However, this did not happen if the plasma came from osteocalcin-deficient mice [174]. Similarly, in humans who have suffered a stroke, patients with higher levels of osteocalcin showed greater improvements on National Institutes of Health Stroke Scale (NIHSS) than did patients with lower osteocalcin levels [182]. This further supports the possibility that osteocalcin levels may impact cognitive function.

Apart from the correlation with full-scale IQ, children with high osteocalcin at four months of age also performed better on the drawing trail test, which requires a well-developed smooth coordination, a function directed by the basal ganglia. This is in analogy with a study by Puig et al. [109], who showed microstructural changes in the basal ganglia in humans based on osteocalcin levels. Furthermore, Guo et al. studied rats with induced Parkinson's disease, these rats showed improved cell survival in the substantia nigra of the basal ganglia and reduced motor deficits after being injected with osteocalcin [176]. So, even though we cannot say anything about causality with the chosen study design, our findings are in line with earlier studies, which increases the likelihood that our results are of physiological relevance.

When it comes to associations between osteocalcin and anxiety or depression-like behavior that has been found for mice [107], we did not find support for this in our cohort of healthy children but power was low, which hampered conclusions. One potential reason for the difference between the studies, might however lie within the complete lack of osteocalcin, as seen in the mice study, or that children in our cohort were too healthy. Nevertheless, we did not find any associations between social, emotional or behavioral problems in relation to osteocalcin levels.

Cord levels were negatively associated with processing speed and fine motor control at four years of age. We cannot explain this finding, and cord levels are more difficult to interpret than blood levels later in life because they depend

on both maternal and child factors as well as the major event of being born. As discussed, cord levels may differ with respect to mode of delivery, gestational age, parity and more, so umbilical cord levels might represent a specific time point, rather than a developmental stage (period of time). The correlation found may therefore be without clinical significance or be by chance, but if this association represents actual causality, it might mean that osteocalcin plays different roles during different phases – similar to what was seen for growth. Furthermore, we found no correlations between osteocalcin at 12 or 36 months of age and neurodevelopment. But, if the results found for osteocalcin at four months and later neurodevelopment are based on early programming, the timings at 12 and 36 months may be too late, with regard to brain development.

5.5 METHODOLOGICAL CONSIDERATIONS

5.5.1 STUDY DESIGN

The aim of this study was to increase knowledge about barriers to effective weight treatment, and to increase knowledge about osteocalcin and its potential association to early growth and neurodevelopment. In choosing a study design, the aim of the study and the research question need to be acknowledged, in order to make appropriate choices. However, one also needs to have in mind ethical, economical and practical considerations. In Study II-IV, we wanted to study factors that could impact later health, which is why the longitudinal design was chosen. A cross-sectional study could have been used for Study I, but, if questionnaires were handed out at five years of age, significant recall bias for the perception of the two-year old weight status would apply. If questions were asked of parents about their two-year-olds and five-year-olds at the same time, we would lack information about the follow-ups in between, and it would not be the same children or parents at the different time points. In Study IV, the study participants were included in both the Small Health and Growth study and an RCT (which explored effects of cord-clamping timings). These two studies had different designs and study protocols, and the randomization for the Cord-clamping study did not impact the protocol for our study. All designs have their innate advantages and drawbacks, and we chose to conduct prospective cohort studies for all included studies. Cohort studies demand long follow-up times, large sample sizes, rigorous follow-ups and are limited by choices made at the start. For instance, as previously mentioned, a limitation of Study I was the wording of the question, “Do you think that your child weighs… too much, too little or just about right”. This wording allows for different interpretations and the question could have been précised with the

words “according to BMI” when this limitation was noticed after the two-year follow-up. However, if we had changed the wording of the question before the five-year follow-up, the two questions would not have been comparable. Another disadvantage of large cohort studies is that they are often costly and need, to deal with dropouts. But, if the condition studied is rather common, one may find preceding risk factors, and the risk for recall bias is low.

One important design question was the number of follow-ups and blood sampling times in relation to developmental stages. Blood sampling was performed at specific time points, chosen to match the follow-up program at the CHC. These time points allow comparison at specific developmental stages, but more time points would have been an advantage. Because of the longitudinal design, however, it was the same children that left blood samples at every time point, thereby limiting the number of sampling times, for ethical reasons. This time point design showed clear differences in osteocalcin levels between different age groups, but also left gaps of knowledge during time points when no sampling was performed. So, this time point design may be considered both a limitation and a strength, in that it revealed a peak in infancy, but may not give information about osteocalcin levels at two or six months of age. Furthermore, blood samples were drawn during office hours; small studies suggest that osteocalcin shows diurnal variation, with peak levels at 8 am and nadir at 4 pm [230]. If so, drawing blood samples in office hours would likely find both the high and low levels, and the significance is likely negligible in a large sample like ours. Additionally, the amount of blood drawn limited the number of analyses, and given that sex hormone analysis needs a relatively large amount of blood, this was not prioritized within the study protocol. Today, with the knowledge gained, the implication for sex hormone analyses during the first year of life has increased and should be considered in future studies.

Furthermore, given the ages under consideration, physical activity or exercise were not assessed in these studies. Exercise increases osteocalcin levels [125, 227, 231]. Both osteocalcin and exercise may enhance muscle function, by promoting uptake and utilization of glucose and fatty acid in skeletal muscle [125]. Exercise enhances brain function similar to osteocalcin pathways and both may ameliorate age related cognitive decline in mice, via BDNF and GPR158 and by modulating RbAp48 [175]. Physical exercise strengthens bone and may reduce menopausal symptoms [232], with or without a linkage to osteocalcin. So, physical activity could be a confounder to the results, but osteocalcin could also be a mediator between physical activity, bone strength, metabolic-, muscle-, and cognitive function.

Below is a description of some of the methodological considerations we have made when it comes to the instruments that were used in the studies that make up this PhD thesis.

5.5.1.1 MEASUREMENTS OF BODY COMPOSITION

We chose to use BMI calculations for body composition assessments in both study I and III. BMI is a simple and fast way to mathematically adjust weight for height that has age- and sex-specific reference values. At a group level, BMI strongly associates to fat mass, but on the individual level, it is not very sensitive and may not differ between weight by muscle or fat mass [34], and its use has therefore been questioned [53]. Furthermore, BMI does not take body fat compartment into account, and may not differ between SAT and VAT. For young children, differences in bodily proportions makes these drawbacks larger than for adults and, although questioned, BMI calculations represent the most widely used tool for measuring body composition as of today. BMI is challenged by WHtR, which may be a better measurement of metabolic risk [38], but WHtR is still not used to the same extent as BMI, which complicates comparison between studies. Another method for assessing body composition is DXA. DXA requires an x-ray machine and interpretation of data, which is why it is more costly compared to BMI and WHtR. However, advantages include low radiation compared to other x-ray methods making it suitable for growing children, short scan times, rapid and easy patient set-up, relatively good measurement precision, stable calibration, etc. [54] Furthermore, it doesn't need any extra shielding, so operators and parents may stay in the room with the child and the child can be awake with fewer requirements to remain still, compared to MRI scans. These are all reasons that DXA has been regarded as the gold standard for body composition measurements [52]. In study III, we chose to study correlations between osteocalcin, BMI, WHtR and DXA-data. Using three ways of assessing body composition makes interpretation difficult. Still, each of the measurements have their advantages and drawbacks, as noted above. Because the research we undertook was at this early stage, the aim was not to determine exactly the coefficient of determination, but to glean as much information as possible, and increase knowledge about osteocalcin and its potential role in growth and in the development of obesity. So, even though DXA may be regarded as the gold standard [52], it is easier to do repeated measurements of BMI or WHtR than repeated DXA scans, and comparison to other studies is simplified when using the same tools. The apparent indecisiveness may also be questioned for mass significance reasons, but the consistency between the findings may also

strengthen the results. This area of research is rather novel, and little is known about human osteocalcin in infancy and childhood. Our studies on osteocalcin levels and growth contribute with new knowledge with a fairly robust sample size. The ability to compare correlations between osteocalcin and BMI as well as osteocalcin and DXA data brings another dimension, and by using both methods, comparison with previous studies is enabled, and a possible explanation is offered as to why previous research has shown conflicting results [159, 229].

5.5.1.2 COGNITIVE ASSESSMENT

For Study IV, the same psychologist did all the assessments, to reduce the inter-individual differences, and to increase the accuracy of the instruments used. The psychologist (BL) was well trained in pediatric assessment, had extensive experience of the instruments used, and used the instruments in a strictly consistent manner.

WPPSI-III

A Swedish version of WPPSI-III was used for Study IV [202]. WPPSI-III is a Wechsler scale designed to measure intellectual abilities through a series of subtests, but has now been largely replaced by the update, WPPSI-IV [233]. WPPSI-III only assesses a certain range of cognitive abilities, has strict age-norms and there are ceiling effects, which is why it may be less exact for children with higher intelligence. The full-scale intelligence quotient was higher than expected in our population, which may or may not mirror the cohort characteristics of higher educated and older parents. Attention difficulties or lack of motivation may impact the result, which is why individual results need careful interpretation; on the group level, however, the Wechsler scales have been used for many years and are some of the most used tests to assess intellectual abilities [234, 235].

MABC-2

The manual dexterity area from the Movement Assessment Battery for Children, Second Edition (MABC-2) [203] included posting coins, threading beads and the drawing trail test. MABC-2 evaluates motor skills and coordination. The test is designed to identify difficulties in motor development and coordination, without being diagnostic. The test is standardized and allows for comparison and identification of potential motor deficits. It is a practical test that may be fun for the child but, compared to the ASQ-III, it is time consuming to administer and score. Furthermore, children learn what they practice, and children aged four may underperform at one domain even if their

motor skill is normal in general. For this reason, interpretation of individual scores needs to take individual circumstances into consideration.

ASQ-III

ASQ-III [204] is a widely used developmental screening tool in which the parent notices whether or not the child has reached an age-related milestone or behavior yet. This gives an overall picture of the child's development at time for the questionnaire. It was also an advantage that the parent could fill out the form at home before the WPPSI-III and MABC-2 at the clinic, which reduced follow-up times for the child. But parents may over- or underestimate their child, and social or cultural bias may affect the result. With these limitations in mind, it is still simple, easy to use, inexpensive, and may identify developmental concerns early. It is also a widely used tool which is an advantage when comparing studies, and was therefore regarded as a good screening tool for this study.

SDQ

The final cognitive assessment tool used for this study was the Strengths and Difficulties Questionnaire (SDQ) [205], assessing social or behavioral problems. SDQ covers the period when the child is 3–4 years of age. It is a three-point ordinal scale that is not centered to the mean (0 = not true, 1 = somewhat true, 2 = certainly true), and is designed as a screening instrument to find difficulties. The total scores on each scale can be used separately to evaluate emotional and or behavioral problems. It is rather quick and easy to fill out, but may be emotionally hard for the parent to fill out accurately. It requires insight and willingness to disclose emotional and behavioral problems, potentially leading to subjective responses. It has, however, been used in numerous settings and different cultures, has been translated into many languages and is considered rather robust in finding difficulties with adequate psychometric properties albeit with some weaknesses when using the different subscales separately [236].

5.5.1.3 LABORATORY ASSESSMENT

Technical aspects of measuring total serum osteocalcin includes separation immediately after collection, storing below -20°C and avoidance of repeated freeze-thaw-samples. The N-terminal mid-region fragment of amino acid 1-43 osteocalcin assay technique (N-MID fragment osteocalcin) is often referred to as total serum osteocalcin. The N-MID fragment osteocalcin uses chemiluminescence technology that requires 50 µL of serum or plasma, and the method has been compared to other enzyme-immunoassays for quantitative

determination of osteocalcin with a correlation coefficient (r) of 1.00. The N-MID technique has a lower variability compared to intact osteocalcin (amino acids 1-49) [237], and it is widely used for total serum osteocalcin measurements [137, 141, 143, 238]. These aspects were practical in relation to other sampling procedures included in the Small H^2GS cohort, and considered to be the best method available. For this study, we decided not to analyze the different forms of osteocalcin, because the technique is less stable, it would be more expensive and the clinical relevance is uncertain [156]. This choice may be regarded as a limitation, in that the lack of information for undercarboxylated osteocalcin we can only draw conclusions about total serum osteocalcin in this PhD project.

5.5.2 CAUSALITY

The longitudinal design of all included studies makes it possible to study early factors with regard to later outcomes, but it also makes interpretation difficult when it comes to confounding factors and causality. Questions about causality, or cause-effect relationship, are common when interpreting observational or epidemiological studies. For this, Hill's criteria for causation [239] may guide in interpretation of findings but may not lead to definite conclusions. For instance, biological gradient, may be useful in some questions but not all, because not all biological associations are dose-response and dose-response may easily arise from a confounding factor. In addition, correlations in human populations may be complex, with more confounding factors making the principles less valuable. Nevertheless, I believe that Hill's criteria allow us to make important considerations that are useful in relation to our studies. For instance, with the background and the knock-out mice studies, linking osteocalcin to both body composition and neurodevelopment becomes likely, even if, at first glance, it may seem unlikely. With the previously published human observations, our results make further investigation reasonable, and even when a complete biological model may not be proposed, there are several attempts. To the best of our knowledge, these are the first studies showing longitudinal patterns of childhood osteocalcin and stating that osteocalcin may have negative correlations to growth and body composition in infancy but positive correlations in childhood may, on first consideration, lessen the probability of causation, but this pattern is not unusual in human medicine, and the result is in analogy with leptin, of which osteocalcin shares pathways. The strength of some of the associations and the consistency between methods used and to earlier studies also adds the probability that there might be causation

between osteocalcin and growth. Regardless, there are too many questions that still need to be answered, and no conclusion about causality is possible to date.

5.5.3 VALIDITY

Other questions of methodological interest are internal and external validity. In short, internal validity relates to how well the study measures what it is supposed to measure. A study with high internal validity has a low uncertainty in the interpretation of the results, and another researcher receiving the same results would most likely draw the same conclusions. External validity relates to the generalizability of the population, setting, period or situation studied.

In talking about validity, different forms of bias should be considered, for instance systematic biases, selection bias, recall bias, and more. In our studies, recall bias was reduced by the longitudinal design, ensuring that questionnaires were filled in at the time for the follow-up. All studies in this thesis are, however, more or less biased when it comes to the study population. For Study I, 69% of all parents of children born in Halland during the time of inclusion chose to participate. After five years, 70% of these children were still enrolled. Participant characteristics did not differ between children enrolled at birth and the ones who were still enrolled at five years of age (see Paper I), which is good, for generalizable reasons. When comparing study participants with the general population of Halland, parental smoking habits, gestational age, birth weight and sex were similar, but when comparing study participants to all children born in Sweden, somewhat fewer fathers were smokers in our material (9.7% vs 11.3%) [200]. Because smoking did not impact our research question, this difference is likely irrelevant to our question. However, perhaps more important was that one of the inclusion criteria was to understand Swedish well enough for informed consent. In Study I, 17.8% had at least one parent born outside of Sweden and 5.5% had both parents born outside of Sweden. This may be compared to 6.6% of the cohort named Identification and prevention of Dietary- and lifestyle- induced health Effects In Children and infantS (IDEFICS) or 12.4% of expected in Sweden at the time [240]. Selection bias is therefore presumed, but, despite this selection bias, parents with foreign backgrounds were represented, with a proportion somewhat in line with other studies [240, 241]. The generalizability of the population may therefore be considered in line with what one can expect for a large cohort. But, there are research questions, in relation to which even small differences in population characteristics may be of greater significance, for example questions about weight perception, which might be a cultural issue [209]. In our study, being

born outside of Sweden was not associated to the agreement between actual weight status and perceived weight status, but the calculation was rough, with dichotomized options. On the other hand, parents from all over the world seem to have difficulties in correctly estimating their child's weight status [206]. Therefore, a potential difference in heritage is likely of minor significance, in that it should be presumed that all parents, independent of heritage, have difficulties in identifying overweight in young children.

When it comes to Study II, population characteristics for the reference population differed from the general population with regard to maternal age at delivery (older) and child birth size (larger) (Study II, Results section). Larger children were expected, because children born pre-term and SGA were excluded. Children born post-term were also excluded, but caesarean sections were overrepresented, and given that one reason for planning a caesarean section is when the child is expected to be large, this likely impacted the results. A higher proportion of caesareans may therefore raise the mean weight, but also the mean age, because caesarean sections are chosen more often the older the mother is [242]. Older age and more highly educated people are also more likely to take part in studies, further aggravating this difference [240].

In the reference population, Study II, more children (30.9%) were born by caesarean section than is recommended in a general population (9-16%) [243]. In the small H^2GS cohort, children were recruited at the maternity ward in 2008-2009. This was an unintended systematic error, leading to selection bias, with underrepresentation of planned caesarean sections (8.1%, 32 of 349) In 2008-2012, 16.8-18.7% of all children born in Halland were born by caesarean section [201]. So, in the second enrollment, in 2010-2012, planned caesarean sections became overrepresented instead. The proportion of caesarean sections vary for different populations [244], and in a population with fewer children born by caesarean section, the reference limit for osteocalcin at birth and three years of age is likely somewhat lower than what we have presented, for reasons yet unknown. When establishing the reference values, we therefore argued whether we should present different reference values, depending on mode of delivery. But, after consideration, we decided that the difference was small, the biological explanation absent and it would make the clinical interpretation more difficult if more factors were taken into account. This is why we chose not to present reference values based on mode of delivery. When knowledge increases in the future, reference limits will likely be somewhat adjusted. Regardless, our reference limits contribute with improved limits for young

children, compared to what was previously known for children born after gestational week 28+0.

In relation to discussing potential selection bias, participating in Study IV meant that the family had to participate in both the Small H^2GS and the RCT, the Cord Clamping Study in 2008. Rigorous study protocols required effort from the families and resulted in participants with older and more highly educated parents, but child characteristics did not differ. One end-point, full-scale IQ was higher than expected in the sample. This higher IQ could possibly be explained by the fact that intelligence is associated with parental educational level [245]. Children with lower full-cale IQ were, however, represented in the sample and the correlation found for osteocalcin and IQ would likely not change, given that it was a linear association that withstood adjustment for parental educational level.

5.5.4 POWER

Another methodological aspect is the question of power – in short, meaning the tests' ability to detect an actual difference between groups. When power is too low, a true difference is not detected. This could be due to small actual differences or a large variability among factors studied in relation to sample size, called Type-II-error. A significance level of 0.05 means that we accept that 1 in 20 results will render statistically significant differences between groups, even when no real difference exists, so-called type-I error. To find true differences, the sample needs be large enough, but this may be difficult, especially when studying children and/or rare diseases. When it comes to blood sampling in healthy children, sample sizes are often low. Our sample size of 551 children may be regarded as fairly large. But for Study IV, the sample size is smaller, with 158 children and several research questions. To find clinically significant differences in neurodevelopment, a sample size of 80-100 was estimated to be sufficient, but somewhat low to find smaller differences. Power calculations were, however, aggravated by the fact that no reliable reference values existed at time and the expected variability of osteocalcin levels were large. After all data was gathered, the variability for osteocalcin at four months of age was still large, but smaller than we had estimated at this age. A limitation of Study IV was therefore the low number of participants because we chose to study differences between the top and bottom quartile groups, which halved the study populations and the power became somewhat low. For calculations based on WPPSI-III and full-scale IQ, the power was sufficient for clinically relevant differences. However, for SDQ calculations, the power was too low.

On the total difficulties score, only eight children scored borderline, and two children scored above cut-off. Therefore, we cannot exclude the possibility that smaller differences exist, even though we did not find any differences in our sample. The study on osteocalcin and neurodevelopment has its limitations; still, to our knowledge, this study is the first to show an association between osteocalcin and neurodevelopment in humans. The results we present need to be repeated, but are in line with experimental findings, which makes the topic even more interesting, and gives reason for further studies.

5.5.5 PRECISION AND ACCURACY

Other questions raised are questions of precision and accuracy related to the instruments used. The precision accounts for the variability of measurements used, while the accuracy relates to how well the test measures what it is supposed to measure. Random errors may reduce precision of an instrument, while systematic errors or choice of instrument more often impacts the accuracy.

The investigation of correlations between a hormone and various endpoints is complex. Although the precision in anthropometric measurements may be considered high, assessing whether osteocalcin relates to fat mass, using weight would have low accuracy. This is because both weight level and osteocalcin level may be confounded by height. Using BMI for calculations will always measure weight for height; associations may relate to body fat, but also to muscle mass, rendering low accuracy and difficult interpretation. Furthermore, mice studies have shown impaired memory function in osteocalcin-deficient mice [107], but tests for intelligence or cognitive function are not completely generalizable between humans and rodents. Using validated instruments for cognitive assessment thereby increases the precision, but because the research is novel and osteocalcin effects on brain function uncertain, our chosen instruments may have low accuracy for osteocalcin effects on brain function.

Beside our choices in study design, the interpretation of the results might be further complicated by generic difficulties in studying children with differences in age and maturity.

5.5.6 ETHICAL CONSIDERATIONS

Besides ethical approvements by the relevant authorities and ensuring receipt of informed consent by the parents, there is always further cause for ethical considerations.

When it comes to Study I, we were interested in knowing whether parents perceived that their child weighed too much, when charts showed that they had overweight or obesity. These are sensitive and provocative questions that give rise to a wide variety of emotions in society [98, 99]. Puhl et al. showed that the terminology used when discussing excess weight, was of major importance, and that parents preferred the word weight or unhealthy weight rather than fat or obese [213]. The word obese, was perceived as blaming and stigmatizing and the authors conclude that language could undermine health discussions and thereby weight management [213]. When asking parents about their child's weight status we decided to use the wording "Do you think that your child weighs…" instead of weight categories like under- or overweight to use a simple, yet precise, but not judgmental or stigmatizing language. Furthermore, I believe that when using a people-first language (one has obesity, one is not obese) this lessens the emotional burden of the word. Despite these perceptions on wordings, I have chosen to use the words overweight and obesity, when refereeing to the risk factor and disease respectively, in this PhD thesis. Childhood obesity, leads to a wide range of complications [32], and shortens life by, on average, 2.5-13 years [65, 66], depending on degree and age. Thereby, ignoring obesity in fear of weight stigmatization or negative reactions as a professional or parent will let the child down, and make life more difficult for the child, which would also be unethical. So, even if just lifting the question, and ask parents for their perception on their own child's weight, as early as two years of age may seem unethical at first glance, the work is important, given the fact that parental perception is the starting point for weight management, and that there are significant clinical implications.

When it comes to the follow-ups, questionnaires and anthropometrics were done by the child health services, an integral part of the Swedish health care system that covers the vast majority of all children born in Sweden. This lessens the time spent for follow-ups by the families and contributes to the normalization of the follow-ups. The Small H^2GS, was planned to reduce the number of extra controls and still have follow-ups at important time points, in order to achieve high quality data. Blood sampling from healthy children may

be problematic for ethical reasons, because young children may not give their own consent or understand the basis for the research or procedure. Blood sampling is nevertheless an important part of research that may improve the health care for their own and future generations. To reduce the possible negative experience for the child, blood samples were drawn from cord blood and followed EMLA® local anesthetics (AstraZeneca, Cambridgeshire, UK) at 4, 12, 36 and 60 months by experienced nurses. In addition, oral glucose was administered as a distraction, if needed, for blood sampling at four months of age. The last follow-up with blood samples was performed at the age of five, while DXA scans were performed at the age of eight. For obvious reasons, children were not asked for their consent at enrollment and because children need to reach a certain level of maturity (age 12 is often used in practice) to take an active stand, informed consent was not obtained from the children for these PhD studies. However, with increased maturity, children could always decline participation.

To present this PhD project and continue with follow-ups for the H^2GS cohort is a way of utilizing and giving value for all the time spent and possible pain experienced by the children and their families during data collection. It may also be considered a good cause for the children, who may feel proud to participate in research projects for the sake of future development.

6 CONCLUSIONS

Overweight and obesity represent two of the biggest health issues of our times. One aim of this thesis was to increase knowledge about factors that contribute to the development or treatment of obesity on individual or parental level. The first study presented in this thesis showed that the majority of Swedish parents did not perceive that their children with overweight weighed too much, but their perception came more into alignment with actual weight status as the child got older. The low level of coherence between actual and perceived weight status may relate to difficulties in detecting overweight or challenges in acknowledging childhood overweight as a problem. This is an important starting point for health care professionals when they meet parents of children with weight problems, because unrecognized problems do not lead to change.

Previously, on the individual level, there was a lack of sex-specific reference limits for osteocalcin at young age, which hindered conclusions about osteocalcin and growth. This is why we presented reference limits at specific time points during the first years of life. These reference limits showed that osteocalcin presented with specific patterns during the first years of life; patterns that were sex-specific. In the third study, we showed that osteocalcin has programming potential, evidenced by the correlations found between early osteocalcin levels and later height, weight and body composition, respectively. Correlations between osteocalcin and anthropometrics were negative in infancy and positive during childhood. If causality exists, this might indicate disparate actions during different growth stages, but it may also be a sign or a mediator of growth tempo. In childhood, girls' osteocalcin correlated to later fat mass, while boys' osteocalcin correlated to later lean mass. This means that correlations between osteocalcin and body composition may be different between the sexes, and that these differences may not be detected using BMI as the endpoint. Correlations were weak but consistent, despite different methods of assessment and our interpretation is that osteocalcin may be involved in the development of obesity, at least for girls. Lastly, we showed that high osteocalcin levels at four months of age correlated to a more mature motor development and higher scores on full-scale IQ at four years of age. This fourth study becomes more interesting in the light of the findings from the second study, showing that breast feeding, mode of delivery and gestational age were factors that correlated to differences in osteocalcin-levels at four months of age. There are still many questions to be answered, but our results support that osteocalcin may have a programming potential and that there is an axis between bone, adipose tissue, gonads and brain.

In summary:

- Parents did not perceive that their child with overweight weighed too much, which is a potential barrier in weight management. High parental BMI and lower educational level were associated with lesser agreement between actual and perceived child weight status but the clinical relevance is low, due to overall low agreement.
- Osteocalcin levels follow an age- and sex-specific pattern during the first years of life, with a peak in infancy for both sexes. Osteocalcin levels correlate to early environmental factors, such as breastfeeding and gestational age and the levels tend to stabilize over time.
- The osteocalcin pattern may reflect or mediate early growth patterns. Early osteocalcin levels may be of significance both when it comes to amplitude and growth tempo and might associate to impending growth, rather than size, in infancy and childhood.
- Osteocalcin levels in serum at four months of age correlate to later intellectual ability and motor development at four years of age.
- Early osteocalcin levels carry information about later body composition and neurodevelopment and may have a programming potential, but more research is needed.
- In the case of causality, early environmental factors like breastfeeding or gestational age may therefore impact later body composition or neurodevelopment through early osteocalcin levels. Osteocalcin may be one factor with importance in childhood weight management.

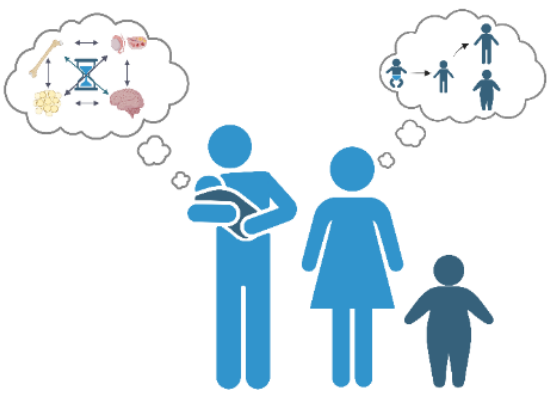

Figure 18, Graphic illustration of the conclusions in this thesis, created in BioRender. Berggren, S. (2024) BioRender.com/b85g409

7 CLINICAL IMPLICATIONS

Overweight and obesity are increasing health problems worldwide. Over one billion humans are obese, and for children the numbers have quadrupled since the 1990s [246]. Obesity often begin at early age, and our study confirms that very few parents living in Halland, Sweden, - regardless of country of birth - recognized overweight or obesity in their children by the age of two and five years. This is important, because no action is taken if the problem goes unnoticed. Health professionals working at child health centers or clinical health centers need to be aware of these premises when trying to reach parents in their clinical work. The likelihood for misperception of child weight status increased if the parent had overweight or a low educational level, but because few parents identified the health problem independent of these factors, we propose that health care professionals should have the preconception that parents, in general, perceive that their child weighs just about right independent of actual weight status.

Furthermore, this thesis contributes to the overall knowledge about human osteocalcin in infancy and early childhood. We propose reference limits for specific time points during the first years of life that may be used by other researchers or in clinical practice. Additionally, we propose that feeding practices, mode of delivery, gestational age, and maternal BMI at gestation are factors, among others, that may impact early osteocalcin levels, and which should be taken into account when interpreting individual osteocalcin samples. Moreover, these factors have possible effects on later growth and neurodevelopment, through early programming. It is known that some people gain weight easier than others, when calory-dense food is available. If there is causality, and if early osteocalcin levels impact this increased likelihood for obesity, knowledge about osteocalcin in growth and body composition may have large clinical implications for future health. However, research about osteocalcin and its association to later body composition or cognition are novel findings, thus far, and without practical application. Given that osteocalcin may be one of the signaling molecules involved in the development of obesity (and metabolic syndrome), further research is warranted and of great importance. Osteocalcin may also be regarded as a possible therapeutic target, when it comes to metabolic disease, which is why increased knowledge should be prioritized. Furthermore, it is theoretically possible that osteocalcin actions may be of importance when it comes to the well-known side effects of corticosteroid treatment.

8 FUTURE PERSPECTIVES

The hormone osteocalcin may be involved in human neurodevelopment and in the development of obesity. Osteocalcin is one individual factor that may impact weight management, another factor is parental misperceptions of childhood weight status. With the aim of preventing future childhood obesity and facilitating effective weight treatment, this thesis contributes with novel knowledge. Increasing knowledge of how and why some people develop obesity and learning what we can do in therapeutic ways need to be prioritized. The association between osteocalcin and human cognition may also have future public health implications, in that it seems possible to impact osteocalcin levels with environmental factors. In this PhD thesis, we present results that give some insight into childhood osteocalcin and growth, but the results also highlight the complexity of childhood growth and development. Research on osteocalcin and body composition and neurodevelopment is only in the beginning, and further studies are needed in many areas.

Based on our results, we propose that future studies on osteocalcin should be timed somewhere around two, three, six and nine months of age, to determine when the infant osteocalcin peak occurs, and whether there is a peak for girls. This would also allow for comparison of the timings to later growth and body composition. More time point data during infancy would also allow for reference intervals based on age intervals during the first year of life, thereby filling in the gaps we present. Additionally, we propose studies that explore possible diurnal variations of osteocalcin. There is also a further need for experimental studies to learn more about osteocalcin in relation to muscle-mass and muscle-function. In the future, it would also be an advantage to analyze osteocalcin in relation to sex hormones, to better understand the sex differences seen, and to study whether or not the shift seen between infancy and childhood is related to mini puberty. Previous animal studies have shown a correlation between osteocalcin and anxiety and depression-like behavior, which we could not confirm. Because of the relatively low prevalence of difficulties within this area for preschool children, we propose a case-control design to further investigate this potential connection.

And lastly, given the novelty of these findings we encourage other researchers to repeat our studies in other populations, to confirm the results.

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