Bone and fat tissue in children and adolescents:

studies with focus on osteocalcin

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Cover illustration: DXA measurements of patients with obesity and anorexia nervosa by Bojan Tubić

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To my family

“The scientific man does not aim at an immediate result.

He does not expect that his advanced ideas will be readily taken up.

His work is like that of a planter – for the future.

His duty is to lay the foundation for those who are to come, and point the way.

He lives and labors and hopes. ”

– Nikola Tesla, 1900

ABSTRACT

The general **aim** was to investigate the possible interplay between bone and fat tissue through clinical studies of children and adolescents. Osteocalcin (OC), a bone formation marker, has been proposed to act as a link between bone and energy metabolism in mice, but human data are inconclusive. The specific aims of this thesis were: (i) to clarify the role of OC in relation to weight, with focus on undercarboxylated OC (ucOC) and carboxylated OC (cOC); (ii) to gain insight on how obesity and underweight affect bone and fat tissue in children and adolescents and; (iii) to study the effect of whole body vibration (WBV) on parameters of metabolic syndrome, bone metabolism and body composition in children with obesity. **Methodology**: Children and adolescents aged 2-24 years were included in the four studies. Study I and II were cross-sectional (case-control), and study III and IV were interventional with a 12-week follow-up, of which study IV was a randomized case-control study. Biochemical parameters were examined in all four studies. Bone mass and body composition were assessed by dual-energy X-ray absorptiometry (DXA), peripheral quantitative computed tomography, heel DXA and laser. Methods of intervention were high-energy diet in patients with anorexia nervosa (AN) and WBV in patients with obesity. **Results:** Total OC and ucOC did not differ between normal-weight and overweight subjects; however, overweight subjects had lower cOC levels, and the measured OC forms did not correlate with insulin and glucose. Overweight children had increased bone mineral content (BMC) and bone mineral density (BMD) in comparison with normal-weight children, and there was a positive correlation between BMC, BMD and body mass index standard deviation score. Adiponectin was inversely correlated with BMC and BMD, and was an independent determinant of BMC and BMD. Patients with AN gained in weight and levels of all three forms of OC and BMC increased. The WBV did not result in any anthropometric changes; however, a reduction of sclerostin implies that WBV therapy has direct effects on bone mechanotransduction.
Conclusions: This thesis could not confirm the hypothesis that OC has a positive effect on glucose and insulin homeostasis, although cOC was lower in obese subjects than in normal-weight subjects. The home-based WBV intervention study in young children with obesity did not result in any effect on weight, metabolic parameters or calcaneal bone mass.

Keywords: Osteocalcin, obesity, adiponectin, carboxylation, anorexia nervosa, paediatric, bone turnover markers, bone mass, whole body vibration, muscle
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SAMMANFATTNING PÅ SVENSKA

Ökad inaktivitet och felaktig kost orsakar livstilsrelaterad ohälsa som fetma och insulinresistens. Fetma är bl a associerat med diabetes, hjärt- och kärlsjukdomar. I kontrast till fetma finns anorexia nervosa (AN) med dess allvarliga somatiska och psykiska följder. Syftet med avhandlingen var att undersöka det endokrina samspelet mellan skelett- och fettvävnad genom kliniska studier på barn och ungdomar. Målsättningen var att studera hela spannet av vikttillstånd, från fetma till AN. Experimentella djurstudier och kliniska studier har visat att benformationsmarkören osteocalcin (OC) har en positiv effekt på metabola parametrar som t ex insulin och glukosnivåer. Fokus var att klargöra den föreslagna positiva rollen för OC med inriktning på de olika OC formerna, underkarboxylerat OC (ucOC) och karboxylerat OC (cOC). Vidare undersöktes hur fetma och undervikt påverkar skelett- och fettvävnaden hos unga och slutligen studera vibrationsträning och dess inverkan på muskler, ben och glukosmetabolism på barn med fetma.

I **delstudie I** deltog 28 normalviktiga och 13 överviktiga/obesa barn där vikt, längd, hälbenets bentäthet samt blodprover undersöktes. Bentätheten var högre hos överviktiga jämfört med normalviktiga barn och positivt korrelerat med standardiserat "body mass index". Adiponektin visade negativt samband med bentäthet men det var inte någon skillnad i grupperna avseende OC. I **delstudie II** inkluderades barn (2-9 år), 62 överviktiga/obesa och 46 normalviktiga kontroller, ur den svenska delen av IDEFICS studien. Totalt OC och ucOC visade ingen skillnad mellan grupperna. Karboxylerat OC var lägre hos överviktiga. Totalt OC och cOC var negativt korrelerat till HbA1C. I **delstudie III** studerades 22 patienter med svår AN under 12 veckors intensiv viktuppgångsbehandling. Vikt, längd, blodprover samt bentäthetsmätningar undersöktes vid studiestart och -slut. Totalt OC, ucOC samt cOC ökade men ingen av OC formerna korrelerade med viktförändring eller insulinnivåer. Mängden benmineral ökade. I **delstudie IV** deltog 30 överviktiga barn, 7-17 år (16 kontroller), i en prospektiv, randomiserad, kontrollstudie där ena gruppen genomgick vibrationsträning under 12 veckor. Vikt, längd och blodprover insamlades. Bentätheten i hälen, muskelstyrka och balans mättes. Vikten och bentätheten var oförändrad men balansen förbättrades.

Sammanfattningsvis har dessa studier inte kunnat konfirmera en positiv metabola roll för OC vid fetma och insulinresistens. Noterbart är att karboxylerat OC var lägre hos överviktiga.

SUMMARY IN SERBIAN - NAUČNI REZIME

Povećana neaktivnost i nepravilna ishrana dovodi do zdravstvenih problema uzrokovanih faktorima životnog stila kao što su gojaznost i rezistencija na insulin. Gojaznost uzrokuje dijabetes i kardiovaskularne bolesti. Suprotno od gojaznosti je anoreksia nervoza (AN) sa ozbiljnim somatskim i psihološkim posledicama. Cilj ovog naucnog rada sprovodenog kliničkim studijama nad decom i adolescentima je bio da se ispita endokrinološka interakcija između koštanog i masnog tkiva. Namera je bila da se prouče dve grupe pacienata, gojaznih i anorektični. Eksperimentalna istraživanja nad životinjama i kliničke studije su pokazale da osteokalcin (OC), marker formiranja kostiju, ima pozitivan efekat na metaboličke parametre poput nivoa insulina i sećera u krvi. Ideja je bila da se potvrdi predpostavljeni pozitivni uticaj OC sa naglaskom na različite oblike OC (nedovljno karboksiliran OC (ucOC) i karboksiliran OC (cOC)). Potom je ispitivano kako gojaznost i pothranjenost utiču na kosti i masno tkivo kod mladih kao i uticaj treninga sa vibracijama na mišiće, kosti i metabolizam sećera kod gojazne dece. U **studiji I** je uključeno 28 dece sa normalnom težinom i 13 gojazne dece sa merenim parametrima: težina, visina, gustina petne kosti i uzorak krvi. Gustina kosti bila je veća kod gojaznih u poređenju sa decom normalne težine i pokazala je pozitivnu korelaciju sa standardizovanim "indeksom telesne mase". Adiponektin je pokazao negativnu korelaciju sa koštanom gustinom. OC se nije razlikovo u grupama. U **studiji II** uključeno je 62 gojazne i 46 dece sa normalnom težinom kao kontrolna grupa (uzrast 2-9 godina stari), izabrana iz IDEFICS studije u Švedskoj. Nivo ukupnog OC i ucOC se razlikovo između grupa. Nivo karboksiliranog OC je bio niži kod gojaznih. Ukupni OC i cOC je pokazao negativnu korelaciju sa HbA1c. U **studiji III** ispitivalo se 22 bolesnika sa teškom AN tokom 12 nedelja intenzivnog režima ishrane za povećane telesne težine. Težina, visina, uzorak krvi i merenje gustine kostiju ispitivani su na početku i kraju studije. Nivo ukupnog OC, ucOC i cOC je bio povećan, ali nijedan od oblika OC nije pokazao korelaciju sa promenom težine ili nivoom insulina. Nivo mineralnog koštanog sadržaja je bio povišen. U **studiji IV** ispitivano je 30 gojazne dece (uzrast 7-17 godina), od kojih su 14 bila kontrolna grupa, u prospektivnoj, randomiziranoj, kontroliranoj studiji. Grupa sa16 dece je bila podvrgnuta treningu sa vibracijama u toku 12 nedelja. Mereni parametri su: težina, visina i uzorci krvi, gustina petne kosti, mišićna snaga i ravnoteža. Gustina petne kosti i težina je bila nepromenjena, dok je ravnoteža bila poboljšana. Kao **zaključak**, ove studije nisu mogle da potvrde pozitivni metabolički efekat OC na gojaznost i insulinsku rezistenciju. Zapažen je niži nivo osteokalcina kod gojazne dece.

SUMMARY IN SERBIAN- НАУЧНИ РЕЗИМЕ

Повећана неактивност и неправилна исхрана доводи до здравствених проблема узрокованих факторима животног стила као што су гојазност и резистенција на инсулин. Гојазност узрокује дијабетес и кардиоваскуларне болести. Супротно од гојазности је анорексиа нервоза (АН) са озбиљним соматским и психолошким последицама. Циљ овог науцног рада спроводеног клиничким студијама над децом и адолесцентима је био да се испита ендокринолошка интеракција између коштаног и масног ткива. Намера је била да се проуче две групе пацијената, гојазних и аноректични. Експериментална истраживања над животињама и клиничке студије су показале да остеокалцин (ОЦ), маркер формирања костију, има позитиван ефекат на метаболичке параметре попут нивоа инсулина и сећера у крви. Идеја је била да се потврди предпостављени позитивни утицај ОЦ са нагласком на различите облике ОЦ (недовољно карбоксилиран ОЦ (уцОЦ) и карбоксилиран ОЦ (цОЦ)). Потом је испитивано како гојазност и потхрањеност утичу на кости и масно ткиво код младих као и утицај тренинга са вибрацијама на мишиће, кости и метаболизам сећера код гојазне деце. У **студији I** је укључено 28 деце са нормалном тежином и 13 гојазне деце са мереним параметрима: тежина, висина, густина петне кости и узорак крви. Густина кости била је већа код гојазних у поређењу са децом нормалне тежине и показала је позитивну корелацију са стандардизованим "индексом телесне масе". Адипонектин је показао негативну корелацију са коштаном густином. ОЦ се није разликово у групама. У **студији II** укључено је 62 гојазне и 46 деце са нормалном тежином као контролна група (узраст 2-9 година стари), изабрана из ИДЕФИЦС студије у Шведској. Ниво укупног ОЦ и уцОЦ се разликово између група. Ниво карбоксилираног ОЦ је био нижи код гојазних. Укупни ОЦ и цОЦ је показао негативну корелацију са ХбА1ц. У **студији III** испитивало се 22 болесника са тешком АН током 12 недеља интензивног режима исхране за повећане телесне тежине. Тежина, висина, узорак крви и мерење густине костију испитивани су на почетку и крају студије. Ниво укупног ОЦ, уцОЦ и цОЦ је био повећан, али ниједан од облика ОЦ није показао корелацију са променом тежине или нивоом инсулина. Ниво минералног коштаног садржаја је био повишен. У **студији IV** испитивано је 30 гојазне деце (узраст 7-17 година), од којих су 14 била контролна група, у проспективној, рандомизираној, контролираној студији. Група са 16 деце је била подвргнута тренингу са вибрацијама у току 12 недеља. Мерени параметри су: тежина, висина и узорци крви, густина петне кости, мишићна снага и равнотежа. Густина петне кости и тежина је била непромењена, док је равнотежа била побољшана.

Као **закључак**, ове студије нису могле да потврде позитивни метаболички ефекат ОЦ на гојазност и инсулинску резистенцију. Запажен је нижи ниво остеокалцина код гојазне деце.

LIST OF PAPERS

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

I. Relation between bone mineral density, biological markers and anthropometric measures in 4-year-old children: a pilot study within the IDEFICS study

Tubić B, Magnusson P, Swolin-Eide D, Mårild S; IDEFICS Consortium. *International Journal of Obesity (London) 2011; 35(Suppl 1): S119-124.*

II. Different osteocalcin forms, markers of metabolic syndrome and anthropometric measures in children within the IDEFICS cohort

Tubic B, Magnusson P, Mårild S, Leu M, Schwetz V, Sioen I, Herrmann D, Obermayer-Pietsch B, Lissner L, Swolin-Eide D; IDEFICS consortium. *Bone 2016; 84: 230-236.*

III. Increased bone mineral content during rapid weight gain therapy in anorexia nervosa

Tubić B, Pettersson C, Svedlund A, Bertéus Forslund H, Magnusson P, Swolin-Eide D. *E-published. Hormone and Metabolic Research, DOI: 10.1055/s-0042-115304.*

IV. Whole body vibration intervention: a randomized, prospective, controlled study in children with obesity

Tubić B, Zeijlon R, Wennergren G, Mårild S, Dahlgren J, Magnusson P, Swolin-Eide D. Manuscript submitted.

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Additional relevant papers not included in this thesis.

I. Description of an intensive nutrition therapy in hospitalized adolescents with anorexia nervosa.

Pettersson C, **Tubić B**, Svedlund A, Magnusson P, Ellegård L, Swolin-Eide D, Bertéus Forslund H.
Eating Behaviors 2016; 21: 172-178.

II. Vitamin D status in young Swedish women with anorexia nervosa during intensive weight gain therapy.

Svedlund A, Pettersson C, **Tubić B**, Magnusson P, Swolin-Eide D. *European Journal of Nutrition 2016 E pub ahead of print. In press.*

III. The IDEFICS validation study on field methods for assessing physical activity and body composition in children: design and data collection.

Bammann K, Sioen I, Huybrechts I, Casajus JA, Vicente-Rodriguez G, Cuthill R, Konstabel K, **Tubić B**, Wawro N, Rayson M, Westerterp K, Mårild S, Pitsiladis YP, Reilly JJ, Moreno LA, De Henauw S. *International Journal of Obesity (London) 2011; 35(Suppl 1): S79-87.*

IV. The relationship between paediatric calcaneal quantitative ultrasound measurements and dual energy X-ray absorptiometry (DXA) and DXA with laser (DXL) as well as body composition.

Sioen I, Goemare S, Ahrens W, De Henauw S, De Vriendt T, Kaufman JM, Ottevaere C, Roggen I, Swolin-Eide D, **Tubić B**, Vyncke K, Mårild S. *International Journal of Obesity (London) 2011; 35(Suppl 1): S125-30.*

V. Validation of anthropometry and foot-to-foot bioelectrical resistance against a three-component model to assess total body fat in children: the IDEFICS study.

Bammann K, Huybrechts I, Vicente-Rodriguez G, Easton C, De Vriendt T, Marild S, Mesana I.M., Peeters M.W., Reilly J.J., Sioen I, **Tubić B**, Wawro N, Wells J, Westerterp K, Pitsiladis Y, Moreno LA. *International Journal of Obesity (London) 2013; 37(4): 520-6.*

CONTENT

ABBREVIATIONS

ALP	alkaline phosphatase
AN	anorexia nervosa
BMAD	bone mineral apparent density
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
BTM	bone turnover marker
cOC	carboxylated osteocalcin
CTX	cross-linked carboxy-terminal telopeptide of type I collagen
CV	coefficient of variation
DPA	dual-photon absorptiometry
DXA	dual-energy X-ray absorptiometry
DXL	dual-energy X-ray absorptiometry and laser
HA	hydroxyapatite
HOMA	homeostatic model assessment
HR-QCT	high-resolution quantitative computed tomography
IDEFICS	Identification and prevention of Dietary- and lifestyle-induced health EFfects In Children and infantS
IGF-I	insulin-like growth factor -I
IR	insulin resistance
NTX	cross-linked amino-terminal telopeptide of type I collagen

OC	osteocalcin
PDGF	platelet-derived growth factor
PICP	type I procollagen carboxy-terminal propeptide
PINP	type I procollagen intact amino-terminal propeptide
pQCT	peripheral quantitative computed tomography
SDS	standard deviation score
SPA	single-photon absorptiometry
SSI	strength strain index
ucOC	undercarboxylated osteocalcin
WBV	whole body vibration

In the text, *bone mass measurements* will be used as an umbrella term for BMD, BMC and BMAD

1 INTRODUCTION

1.1 Childhood obesity

The prevalence of obesity in the world has more than doubled since 1980, with as many as 39% of adults classified as overweight and 13% classified as obese. Worldwide, 42 million children were overweight or obese in 2013 [1]. Never before have so many children and adolescents been obese, although the increase in prevalence seems to be levelling off worldwide [2] and stabilizing in Sweden[3]. Obesity is a disease in itself but it is also a key risk factor for other non-communicable diseases (NCD) such as cardiovascular disease, type 2 diabetes, musculoskeletal disorders and dental disease. In 2001, the proportion of global burden of disease that was attributed to NCDs was 46%; this proportion is expected to increase to 57% by 2020 and to appear in considerably younger age groups [4].

Overweight and obesity are defined as "abnormal or excessive fat accumulation that may impair health" [1]. There is also an arbitrary definition: for adults, overweight is defined as body mass index (BMI) above 25 kg/m^2 and obesity is defined as BMI above 30 kg/m^2 [1]. While age, sex and genetic susceptibility are non-modifiable, many of the risk factors associated with age and sex can be modified, for example, behavioural factors (diet, physical activity), biological factors (overweight, hyperinsulinaemia) and social (socioeconomic, cultural) factors.

In our society, obese people are often blamed for being irresponsible, lazy and/or undisciplined. But there are also important environmental factors to take into account. There is evidence that when people move to a new environment they may gain in weight [5], for example, moving from one country to another country where obesity is more prevalent. When dealing with obesity, it is important to focus not only on what individuals can do to improve their situation, but also on the whole environment around the individual.

1.2 The IDEFICS study

IDEFICS (Identification and prevention of Dietary and lifestyle-induced health EFfects In Children and infantS) started in 2006 as a European multicentre study. The background to the IDEFICS study was the changed environment for children in Europe with unhealthy dietary habits and a

sedentary lifestyle [6]. The study was in accordance with the WHO strategic directions and recommendations for policy and research about diet, nutrition and prevention of chronic diseases, proposed in 2002 [4]. The IDEFICS study started in 2006 and finished in 2011. The main aim of the study was to investigate the aetiology of diet and lifestyle-related diseases in a large prospective cohort, focusing on overweight and obesity; the secondary aim was to develop, implement and evaluate a community-oriented, population-based intervention programme for primary prevention of obesity in a case-control design setting [7].

At baseline, the prospective cohort comprised of 16 224 children aged two to nine years in eight European countries (Belgium, Estonia, Cyprus, Germany, Sweden, Hungary, Italy and Spain). The Swedish part of the study at baseline consisted of 1 837 children in the Gothenburg area. The study was planned during 2006–2007; thereafter the baseline survey (T0) was performed in 2007-2008. The intervention was executed in 2007–2008 as a case-control design. The follow-up survey (T1) was performed in 2009–2010. During 2010-2011, all the collected data were structured and cleaned before being used in further research. The baseline and follow-up surveys were designed to assess overweight and obesity (using anthropometric measurements and lifestyle questionnaires), musculoskeletal disorders (using qualitative ultrasound examination of the calcaneus) and insulin resistance (using biochemical markers) [7].

1.3 Anorexia nervosa

Anorexia nervosa (AN) is a psychiatric disorder with severe consequences, primarily seen in adolescent girls. The definition of AN according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) criteria [8] is: significantly low body weight, intense fear of gaining weight, altered body image and lack of recognition of the seriousness of the low body weight. The prevalence of AN is approximately 1% among 17-year-old Swedish girls [9]. The average prevalence rate in the world is approximately 0.3%. Incidence rates worldwide are up to 8 per 100 000 people per year, though it must be underlined that even the most well-designed studies underestimate the true incidence [10]. Approximately 10% of AN patients are men [10]. Most patients recover from severe AN but there is a relatively high prevalence of mortality in the group, with a mortality rate of 3% to 18%, depending on the study [11]. AN leads to severe complications, for example, an increased risk of suicide, neuropsychiatric co-morbidities, amenorrhea, hormonal imbalance with potentially lower bone quality and osteoporosis [12, 13].

1.4 Bone tissue

The study of human bone tissue has been of interest since at least the second century of the common era, when Galen of Pergamon (129 CE - 200/216 CE studied the human skeleton in Alexandria, Egypt, and recommended his students to seize every opportunity to do the same [14]. The human skeleton has three distinct functions: firstly, it gives us an upright posture and it is a point of attachment for our muscles, enabling us to move; secondly, the skeleton protects our vital organs, including the brain, heart and lungs; thirdly it serves as a reservoir for calcium, phosphate, lipids and bone marrow. The latest proposed role is a possible endocrine function as a regulator of whole-body glucose metabolism and male fertility [15, 16].

A fascinating characteristic of bone is its unique combination of strength and flexibility. It allows us to walk and at the same time it can tolerate bending, compression and torsion without breaking. To maintain these characteristics, bone is constantly adapting to the physiological and mechanical strains that challenge it.

1.5 The composition of bone

The human skeleton consists of 206 bones, which can be divided into long bones (such as the tibia and femur) and flat bones (such as the cranium and pelvis). The skeleton as an organ consists of cartilaginous joints, calcified cartilage, the marrow space and the mineralized structures including bones. Mineralized bone is made up of cells, vessels and crystals of calcium compounds (hydroxyapatite). Macroscopically there are two kinds of bone tissues: *cancellous* (porous/trabecular) and *cortical* (compact) (Fig 1). Cancellous bone is found in the metaphysis of long bones and in the vertebrae; cortical bone is the hard bone located on the surface. The proportion of cancellous and cortical bone determines the mechanical quality of bone and its resistance to fractures [17]. Bone mass consists of approximately 80% cortical bone and 20% cancellous bone, but the cancellous bone has a much larger area (90% of its total area) exposed to other tissues and bone marrow. This makes the cancellous bone the main target for bone mineral metabolism [17].

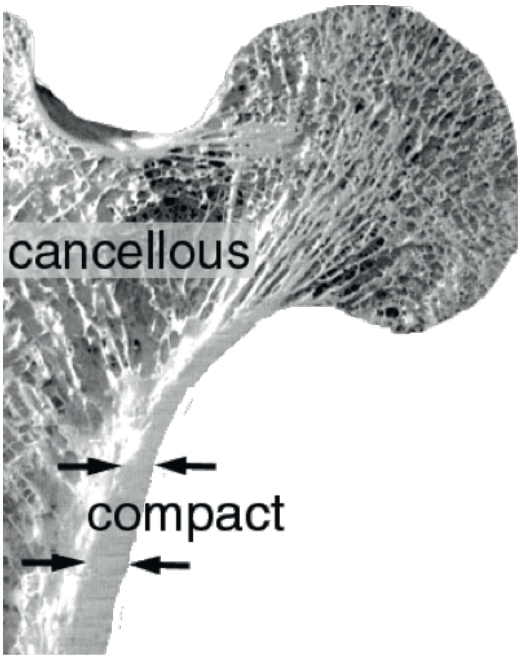

Figure 1. Human femur cervical neck. Cancellous and compact (cortical) bone.
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1.6 Bone cells

There are three types of bone cells: *osteoblasts*, which are responsible for bone formation, *osteocytes*, which are osteoblasts that are trapped in osteoid, and *osteoclasts*, which are responsible for bone resorption [18].

1.6.1 Osteoblasts

Osteoblasts produce bone matrix. They originate from multipotent mesenchymal stem cells, which can differentiate into osteoblasts, chondrocytes, myoblasts, adipocytes or fibroblasts [19]. As the period for matrix production ends, 15% of the osteoblasts are entrapped in the newly produced matrix and become osteocytes. Osteocalcin (OC) is specifically expressed by osteoblasts [16]. Osteoblasts have receptors for, and produce, growth factors such as insulin-like growth factors (IGFs) and platelet-derived growth factor (PDGF). Activity of the osteoblasts is regulated in a paracrine and autocrine manner by these growth factors. Osteoblasts also have receptors for hormones such as parathyroid hormone, thyroid hormone, insulin and growth hormone [18].

1.6.2 Osteoclasts

Osteoclasts are large multinucleated cells derived from hematopoietic cells of mononuclear lineage. The osteoclast is responsible for bone resorption. During the process of bone resorption, the osteoclasts adhere to the bone matrix and move along the bone surface. Osteoclasts resorb bone by enzymatic proteolysis of bone matrix and acidification of hydroxyapatite (HA) crystals, which are found within the sealing zone [18]. Studies have demonstrated receptors on osteoblasts for thyroid hormone, androgens, calcitonin, insulin, IGF-I and PDGF, among other hormones and growth factors [18].

1.6.3 Osteocytes

Osteocytes are differentiated osteoblasts that become surrounded by bone matrix during bone formation. The exact function of osteocytes is not fully understood; however, it has been suggested that they attract osteoclasts to sites where bone remodelling is required, as a response to bone tissue strain or microdamage [20]. Sclerostin, a glycoprotein, has recently been proposed to be a product of osteocytes acting as an inhibitor of bone formation [21]. Sclerostin levels increase with age [22] and sclerostin is reported to be positively associated with BMD [23]. Mechanical loading has been reported to bear an inverse relation to circulating sclerostin levels [24, 25], whereas immobilization shows a positive correlation [26]. Studies have also demonstrated both positive [27] and non-existing [23] correlation between sclerostin levels and fracture risk.

1.7 Bone remodelling

The process of bone remodelling is vital, as the bone is a dynamic organ with continuous defects and microfractures which are repaired by the actions of osteoblasts and osteoclasts. Harold Frost was the first to demonstrate this process [28]. During remodelling, there is a close interplay between osteoclasts and osteoblasts, in which they form a basic multicellular unit (BMU). Between 2% and 5% of cortical bone is remodelled per year. As cancellous bone has a much larger surface-to-volume ratio, it is more actively remodelled than cortical bone. The remodelling cycle can be divided into different phases: resorption, reversal and formation (Fig 2). The resorption phase lasts for two weeks and is the period when osteoclasts break down tissue and form a resorption cavity; the reversal phase lasts for four to five weeks, followed by the formation phase, in which osteoblasts fill the cavity, which takes approximately three to six months [18, 29, 30].

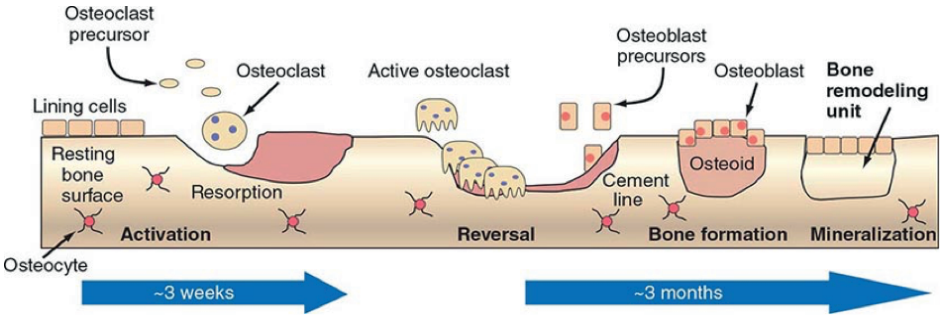

Figure 2. Bone remodelling. (D.L Kasper, A. S. Fauci, S. L. Hauser, D. L. Longo, J. L. Jameson, J. Loscalzo. Harrison´s principles of internal medicine, 19th edition.) © McGraw-Hill Education. All rights reserved.

1.8 Bone growth and peak bone mass

Peak bone mass can be defined in several ways. At the individual level, the concept of peak bone mass can be defined as the maximum amount of bone accrued during young adulthood. At the population level, peak bone mass is attained when bone outcome has reached a plateau or maximum value, or when age-related changes in bone outcome are no longer positive [31, 32] (Fig 3). The age at which different bones reach peak bone mass varies depending on anatomical region [33, 34]. Studies have reported that peak bone mass in the femur is reached at approximately 16–18 years of age in girls [33, 35] while the distal radius reaches peak bone mass at 40 years of age and above in women [34, 35]. There is an uncertainty whether bone mass is positively or negatively affected by fat mass [36]. There are studies where the majority of children in the fracture group are overweight [37, 38]. It is important to understand the interaction between fat and bone when developing future health strategies to optimise peak bone mass and prevent fractures [36].

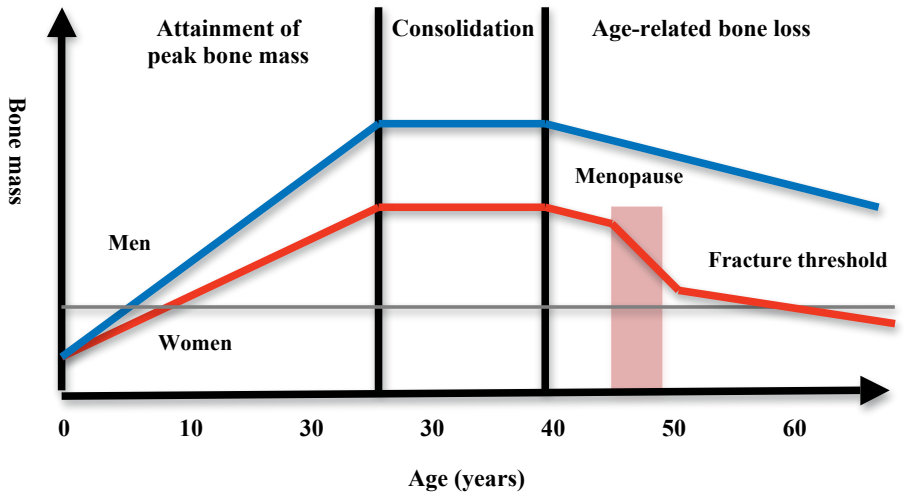

Figure 3. Peak bone mass. Copyright © 1990 Compston JE[39]. The red line represents women and the blue line represents men.

1.9 Bone turnover markers

Bone metabolism mirrors the complex interplay of osteoblasts, osteoclasts and osteocytes. To analyse this interplay, serum and urine assays have been developed. In these assays, biochemical markers are analysed, reflecting the activity of osteoblasts and osteoclasts and the breakdown products of bone tissue. Bone turnover markers (BTM) are divided into markers of bone formation and bone resorption, and are not necessarily tissue- or site-specific (Table 1). The clinical application of these assays is to investigate diseases that alter bone metabolism, for example osteoporosis, to estimate fracture risk, and compliance in order to evaluate pharmaceuticals against the disease in question [40]. The change in levels of BTMs is faster (response within weeks) than the change in BMD that can be measured with, for example, dual-energy X-ray absorptiometry (DXA) or peripheral quantitative computed tomography (pQCT).

1.9.1 Bone formation markers

Bone formation markers can be measured in serum and reflect the different phases of osteogenesis, but they are not all specifically produced by osteoblasts. Osteoid is produced in the early phase of bone formation and 90% of osteoid is type I collagen, which is expressed by osteoblasts. When type I collagen is produced, carboxy-terminal (PICP) and amino-terminal (PINP) propeptides are cleaved of type I procollagen. The circulating levels of these propeptides reflect the amount of newly synthesized type I collagen.

Type I collagen is not specific to bone tissue, as it can also be found in tendons and in skin, although it mainly originates from bone. PINP has been chosen by the International Osteoporosis Foundation as the reference marker for bone formation, due to its stability, assay performance and response to treatment [29]. Another bone formation marker is OC, which is produced by osteoblasts and is bound to HA in the mineralized matrix of bone. A third bone formation marker is bone alkaline phosphatase (ALP), which contributes to bone mineralization [29].

1.9.2 Bone resorption markers

Markers of bone turnover are products of the degradation of type I collagen, non-collagenous proteins and enzymes, which are produced during osteoclast activity. When bone is resorbed, peptide fragments of type I collagen, such as carboxy-terminal and amino-terminal cross-linked telopeptides of type I collagen (CTX and NTX, respectively), are released into the blood. Both can be analysed with automated measurement. The International Osteoporosis Foundation has recommended using CTX as the reference marker for bone resorption [29]. During osteoclast activity, tartrate-resistant acid phosphatase type 5 (TRACP5b) is produced by osteoclasts and reflects the number of osteoclasts [29].

Table 1. Bone turnover markers.

Bone formation markers	**Bone resorption markers**
Type I procollagen carboxy-terminal propeptide (PICP) Type I procollagen intact amino-terminal propeptide (PINP)	Telopeptides of type I collagen (C-terminal: CTX; N-terminal: NTX)
Osteocalcin (OC)	Tartrate-resistant acid phosphatase (TRACP5b)
Alkaline phosphatase (Total ALP and bone ALP)	

1.10 Osteocalcin

Osteocalcin was first isolated by Hauschka et al. [41] in 1975, and confirmed by Price et al. in 1976 [42]. OC was characterized as a gamma-carboxyglutamic acid-containing (Gla) protein from bone; it was found to bind strongly to HA and was confirmed as an important factor in the bone extracellular matrix. Osteocalcin is also known as bone Gla protein (*BGLAP*) and is the most abundant osteoblast-specific non-collagenous protein [43]. OC is a small protein of 49 amino acids in humans and 46 amino acids in mice. It is synthesized in the osteoblast as a pre-promolecule (Fig 4).

Post-translational vitamin K-dependent gamma carboxylation occurs where three glutamic acid (Glu) residues (GLU13, GLU17 and GLU20) are carboxylated into Gla residues by gamma carboxylase. This gamma carboxylation results in a greater affinity for calcium and HA. Eventually, pro-OC is cleaved into carboxylated and undercarboxylated OC. The gamma carboxylation of the three Glu residues is important for the structure and function of the fully cOC, enabling it to bind HA with high affinity and to regulate bone mineral maturation. After the intracellular cleavage, OC is secreted from the osteoblast cell [43]. OC exists in serum in fully carboxylated, partially carboxylated and undercarboxylated forms [44-46]. Carboxylated OC is deposited into the bone extracellular matrix together with calcium and partly released into the circulation, whereas ucOC (where 0-2 Glu residues are carboxylated) is mainly released into the circulation [47]. In 1984, Brown et al. demonstrated evidence of OC acting as a bone formation marker, which could be used to evaluate the treatment of postmenopausal osteoporosis [48].

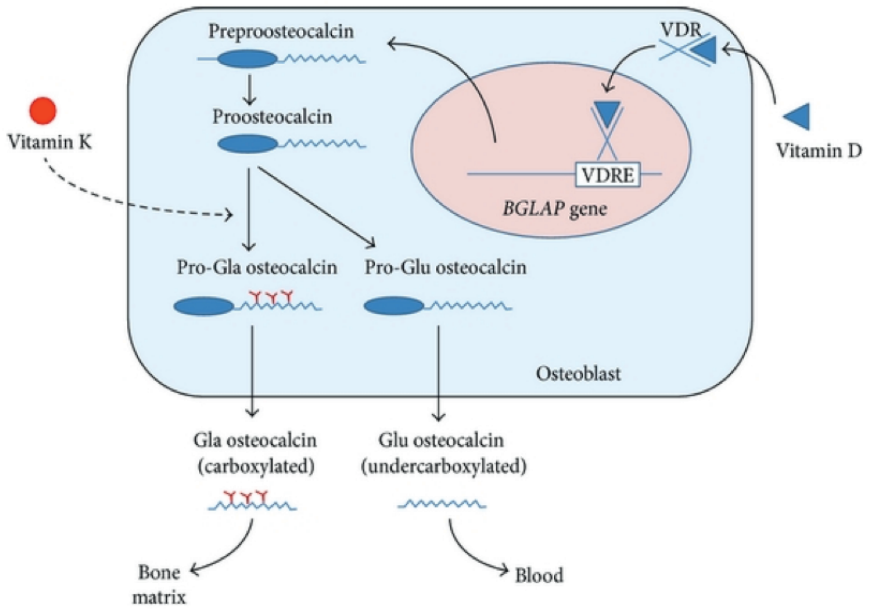

Figure 4. Synthesis of OC in osteoblasts. Vitamin D stimulates the transcription of the BGLAP gene. The preproosteocalcin is cleaved to proosteocalcin. A proportion of proosteocalcin is then carboxylated in a vitamin K-dependent process. The final products are carboxylated and undercarboxylated OC that are released from osteoblasts in a calcium-dependent process. Carboxylated OC is mainly involved in the mineralization of bone matrix while undercarboxylated OC is released into the circulation. VDR=vitamin D receptor, VDRE=vitamin D responsive element, Gla=gamma-carboxyglutamic acid-containing protein, Glu=undercarboxylated OC. Copyright © 2013 Aurora Patti et al.[49].

1.11 The endocrine role of bone tissue, with focus on energy metabolism

Until recently, the endocrine role of bone has been regarded as a regulator for calcium- phosphorus homeostasis and as a source of hematopoietic cells [15]. During the last 15 years, scientists have studied and proposed that bone acts as an endocrine organ in a wider context.

This research field was initiated by Gerard Karsenty and his group with a publication in 1996 in which Ducy et al. [50] generated an OC-deficient mouse model and demonstrated that the mice developed a phenotype marked by higher bone mass and bone with improved quality. Absence of OC led to increased bone formation and OC was proposed as a negative regulator of bone formation. In 2000, Ducy et al. [51] proposed a common regulation of bone mass, body weight and gonadal function. They studied leptin-deficient and leptin-receptor-deficient mice that were both obese and hypogonadal, and they demonstrated that both mouse models had increased bone formation.

Major progress was made by Lee et al. [52] when they demonstrated that mice null for *Esp* (also known as Ptprv, a gene encoding the osteotesticular protein tyrosine phosphatase, OST-PTP) were hypoglycaemic and protected from obesity and diabetes because of an increase in β-cell proliferation, insulin secretion and insulin sensitivity (Fig 5). Their phenotype was fully corrected by crossing *Esp*-null mice with OC heterozygous mice. Further results from the same study presented *in vitro* experiments where OC-producing osteoblasts enhanced insulin production by pancreatic islets, insulin sensitivity and adiponectin expression in adipocytes. In contrast, $OC^{-/-}$ knockout mice, which did not have any OC production, presented with obesity and glucose intolerance. The positive effects were attributed to ucOC. Carboxylated OC and ucOC are proposed to have distinct functions. The two OC forms have different negative charges and calcium-binding properties, which could explain their diverse biological functions [47]. Ferron et al. [53] reported in 2008 that OC *in vitro* induces expression of insulin genes, increase β-cell proliferation and induces adiponectin expression (Table 2). They could also demonstrate decreased OC levels, increased insulin levels and decreased fat pad mass in mice receiving OC through an implanted pump. In 2010 Ferron et al. [54] conducted further studies of OC, in which they demonstrated that insulin signalling in osteoblasts enhances OC activity through a promotion of bone resorption in osteoblasts leading to an increased decarboxylation of OC resulting in increased metabolic activity.

Karsenty´s research group expanded its studies of OC based on the hypothesis that bone mass, body weight and gonadal function are regulated by common pathways [51]. Oury et al. [55] established in 2011 that ucOC favours male fertility by promoting testosterone synthesis by Leydig cells. Otani et al. [56] demonstrated both *in vitro* and *in vivo* (in mice) in 2015 that ucOC stimulates adipocytes to produce adiponectin. Oury et al. [57] also suggested that, in mice, OC influences brain development and function by preventing anxiety and depression as well as favouring learning and memory. These effects are the result of OC suppression of GABA biosynthesis and favour the expression of serotonin and catecholamine synthesis.

When trying to elucidate the function of a hormone, it is of great importance to demonstrate which receptor it binds to. In the case of OC, the receptor is proposed to be a G-protein coupled receptor: GPRC6A [58], although the structure and function of the receptor has not yet been reported [47]. There have been some studies that contribute to the theory that GPRC6A is a potential OC receptor [58-60], but other groups have not demonstrated evidence in favour of this theory [61, 62]. If GPRC6A is to be confirmed as the OC receptor in the future, then most certainly more functions will appear for OC, because GPRC6A is expressed widely [63]. So far, no other receptor has been identified as a potential receptor for OC.

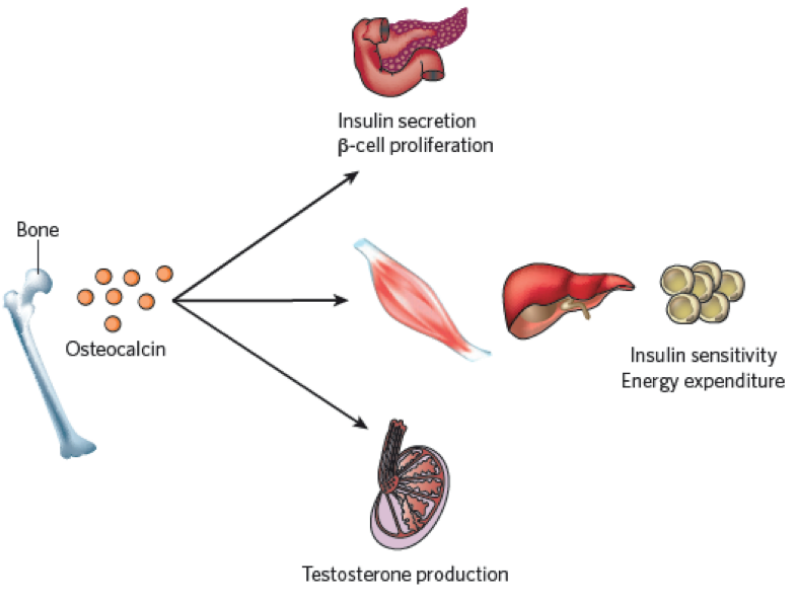

Figure 5. Different functions of OC. It is suggested that undercarboxylated OC stimulates β-proliferation and insulin secretion in the pancreas, energy expenditure in muscle, and insulin sensitivity in muscle, liver and adipose tissue. Through the activation of the receptor GPRC6A it also promotes testosterone synthesis in Leydig cells in the testis, leading to increased male fertility. Copyright © 2012 Karsenty et al. [64].

Table 2. Associations between bone turnover markers and glucose metabolism and adiposity in children and adults.

Measure of glucose metabolism and adiposity	N	Total OC	ucOC	cOC	Other	Measures of bone resorption	Reference
HOMA-IR	2493 M+F	inverse [a]	- [b]	-	-	-	Saleem et al., 2010[65]
	1597 M	inverse	inverse	-	-	no assoc. (TRACP)	Iki et al., 2012[66]
	1010 M	inverse	-	-	-	-	Kindblom et al., 2009[67]
	580 M+F	inverse	-	-	-	-	Gravenstein et al., 2011[68]
	348 M+F		no association	inverse	-	-	Shea et al., 2009[69]
	141 M (children)	no association	-	-	-	-	Jürimäe et al., 2015[70]
	106 M+F (children)	no association	inverse	-	-	-	Boucher-Berry et al., 2012[71]
	36 F (children)	no association	-	-	positive (PICP)	positive (NTX)	Misra et al., 2007[72]
Insulin sensitivity	ESP-/ESP- mouse		positive	-			Lee et al., 2007[52]
B-cell proliferation	mouse		positive	-			Ferron et al., 2008[53]
Insulin (fasting)	2493 M+F	inverse	-	-	-	-	Saleem et al., 2010[65]
	1010 M	inverse				positive (PINP)	Kindblom et al., 2009[67]
	380 M+F	inverse	-	-	-	no assoc. (NTX)	Pittas et al., 2009[73]
	68 M+F	no association	-	no association	-	no assoc. (PINP)	Viljakainen et al., 2014[74]
	140 M+F (children)	no association	no association	inverse	-	-	Pollock et al., 2011[75]
	106 M+F (children)	no association	no association	-	-	-	Boucher-Berry et al., 2012[71]
	36 F (children)	no association	-	-	positive (PICP)	positive (NTX)	Misra et al., 2007[72]
Insulin	mouse		positive	-			Ferron et al., 2008[53]
Glucose (fasting)	2493 M+F	inverse	-	-	-	-	Saleem et al., 2010[65]
	1597 M	inverse	inverse	-	-	no assoc. (TRACP)	Iki et al., 2012[66]
	1010 M	inverse	-	-	-	no assoc. (PINP)	Kindblom et al., 2009[67]
	328 M+F	inverse	-	-	-	-	Kanazawa et al.,2009[76]
	290 M+F	no association	-	-	-	-	Buday et al., 2013[77]
	141 M (children)	no association	-	-	-	-	Jürimäe et al., 2015[70]
	68 M+F	no association	-	no association	-	no assoc. (PINP)	Viljakainen et al., 2014[74]

Measure of glucose metabolism and adiposity	N	Total OC	ucOC	cOC	Other	Measures of bone resorption	Reference
	140 M+F (children)	no association	no association	no association	-	-	Pollock et al., 2011[75]
	106 M+F (children)	positive	no association	-	-	-	Boucher-Berry et al., 2012[71]
	64 M+F (obese)	inverse	-	-	no assoc. (PINP)	-	Iglesias et al., 2011[78]
Adiponectin	2493 M+F	positive	-	-	-	-	Saleem et al.,2010[65]
	348 M+F	positive	no association	positive	-	-	Shea et al., 2009[69]
	149 F	positive	-	-	-	-	Kanazawa et al.,2009[76]
	179 M	no association	-	-	-	-	Kanazawa et al.,2009[76]
	141 M (children)	no association	-	-	-	-	Jürimäe et al., 2015[70]
	103 M+F	-	-	inverse	-	-	Prats-Puig et al., 2010[79]
% Body fat	443 M+F	inverse (in F)	-	-	-	-	Shea et al., 2010[80]
	179 M	inverse	-	-	-	-	Kanazawa et al.,2009[76]
	106 M+F (children)	inverse	no association	-	-	-	Boucher-Berry et al., 2012[71]
	79 M + F (children)	inverse	-	-	-	-	Wang et al., 2014[81]
BMI	2493 M+F	inverse	-	-		-	Saleem et al., 2010[65]
	380 M+F	inverse	-	-	-	no assoc. (NTX)	Pittas et al., 2009[73]
	141 M (children)	Inverse	-	-	-	-	Jürimäe et al., 2015[70]
	106 M+F (children)	inverse	inverse (in M)	-	-	-	Boucher-Berry et al., 2012[71]
	103 M+F (children)	-	-	positive	-	-	Prats-Puig et al., 2010[79]
	83 M	-	no association	no association	no association	-	Foresta et al., 2010[82]
Fat mass	mouse		inverse	-			Ferron et al., 2008[53]

Modified from Booth et al., 2013, Nature Rev Endocrinol.[83]

OC=osteocalcin, ucOC=undercarboxylated osteocalcin, cOC=carboxylated Osteocalcin, HOMA-IR=homeostatic model assessment - insulin resistance, F=female, M=male.

[a] Only statistically significant ($P<0.05$) associations (positive or inverse) are shown.

[b] No measurements were reported.

1.12 Adiponectin

Adiponectin was first discovered by Scherer et al. [83] in 1995. The first name given was Acrp30, (adipocyte related complement protein of 30 kDa) which was later renamed to adiponectin. Adiponectin is a protein made only by adipocytes and its secretion is enhanced by insulin. Even in the first publication, adiponectin (Acrp30) was proposed as a possible factor in energy homeostasis as well as in fat and glucose metabolism [83]. Since then, more than 15 000 papers containing adiponectin in the title or abstract have been published. Adiponectin has several important functions but the primary ones are its insulin-sensitizing qualities in peripheral tissue and its protective effects against inflammation and apoptosis [84]. A number of studies have demonstrated an inverse association between adiponectin and insulin levels, glucose concentration and obesity [85-87]. Yamauchi et al. [88] have demonstrated in mice studies that administration of adiponectin decreases insulin resistance and Gao et al. [89] have suggested a causal relationship between adiponectin and insulin resistance. The endocrine role of OC has been described in numerous publications in the same context as adiponectin, where the two proteins have been proposed to interact. OC and its positive metabolic actions have been suggested to be partly mediated through an induction of adiponectin production in adipocytes and a decrease in adipocyte size [52, 56].

1.13 Bone mass measurements and body composition

In 1895, Wilhelm Conrad Röntgen discovered what he called “X-rays” which paved the way for the first X-ray machines. In 1901 he received the first Nobel prize in physics. With the invention of the radiographic technique it became possible to visualize different qualities of bone, for example osteoporosis. In 1959, a workshop was held on bone densitometry in Bethesda, USA. The organizing researchers conducted an overview of all existing literature, which was published in 1962 by Garn [90]. They were surprised by the volume of research that had been conducted (125 papers during the period 1897–1961). In 1963, Cameron and Sorensen [91] described the single-photon absorptiometry (SPA) method, which was also described by Nilsson in 1966 [92]. This method was peripheral and enabled calculation of the mineral content in grams of calcium hydroxyapatite per centimetre length of bone. Later on, dual-photon absorptiometry (DPA) was developed from the SPA method. DPA uses two photon sources and makes it

possible to measure the hip and spine, because they are located centrally in the body. Subsequently, DPA was replaced with DXA.

1.13.1 DXA

Dual-energy X-ray absorptiometry (DXA) was first described by Cullum et al. [93] in 1987 and is now the gold standard of clinical bone densitometry techniques. One main difference compared to the DPA technique is that the photons are produced from a low-dose X-ray tube instead of from a source of radionuclides. DXA sends out X-rays in two distinct energy levels and is able to measure two different tissue components, bone and soft tissue, where it is assumed that the relationship between lean soft tissue and adipose tissue is constant. DXA is a two-dimensional X-ray method in contrast to three-dimensional methods such as pQCT [94].

DXA scans are used to measure BMD at hip, spine, total body and forearm. The three major clinical roles of the DXA scan are to diagnose osteopenia, to assess the patient's risk of fracture and to monitor the effect of treatment [95]. Furthermore DXA is used to analyse body composition such as "fat mass", "lean mass" or "fat-free soft tissue" [96].

1.13.2 pQCT

Peripheral quantitative computed tomography (pQCT) is primarily used in research, although the technique is also becoming more popular in the clinical setting (Fig 6). The advantage of this method is its ability to measure bone geometry and volumetric bone density (three-dimensional X-ray method). It is a peripheral method which most commonly measures sites in the appendicular skeleton, for example, the tibia and radius. Recently, high-resolution quantitative computed tomography (HR-QCT) has been introduced, mainly in research. The HR-QCT technique is an improvement on the QCT technique, primarily because of its higher resolution, allowing estimation of micro-architectural properties such as cancellous bone size and number, and because of its lower radiation dose [97].

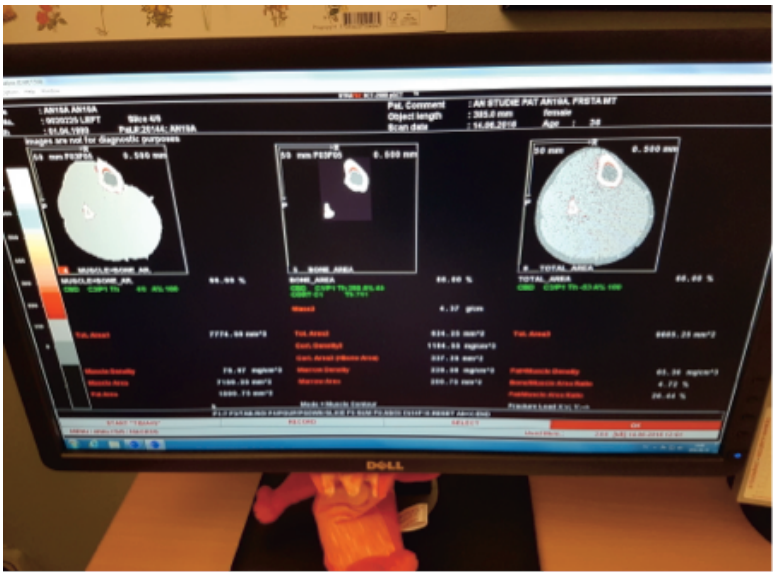

Figure 6. Patient during a pQCT of tibia (above) and output on computer screen (below). With permission from the patient.

1.13.3 DXL Calscan

The dual-energy X-ray absorptiometry and laser technique (DXL) Calscan system (Fig 7) is based on the DXA method with the addition that the total width of the heel is also measured with laser and used in the calculation of BMD [98]. With the DXL Calscan system (paediatric version) calcaneal BMD [99], and a new parameter called bone mineral apparent density (BMAD) is measured [100, 101]. The DXL Calscan system predicts osteopenia well and the correlation with the whole-body DXA method is high [99, 102, 103]. The DXL Calscan has been used to diagnose osteoporosis in adults and children[100] and it is used in conjunction with measurements using axial DXA technology[103, 104].

Figure 7. DXL Calscan bone densitometer.

The available methods have their strengths and limitations. A summary of several of the methods is given in Table 3.

Table 3. Summary and comparison of different bone measurement techniques.

Technique	Site	Examination time (min)	Radiation dose (μSv)	Precision (CV%)	Advantages	Limitations
DXA	Lumbar spine Total body Proximal femur	3–10 per bodypart	0.4–4 1–2	<1 1-2 0.2-5.4	1. High precision 2. Paediatric reference data available 3. Low radiation dose 4. Rapid scan time 5. Can assess body composition	1. Size-dependent measurements 2. Integral measurement of cancellous and cortical bone 3. Sensitive to body composition changes
DXL	Calcaneus	<5	<0.2 (adult) <0.12 (children)	1.2 2.4-9.8 (ages 2-7)	1. See DXA 1–4 2. Portable machine 3. Short measuring time (≈ 2 min)	1. See DXA 1–2 2. Only calcaneus can be measured
pQCT	Radius Tibia	5–15	< 1.5–4 per scan < 1.5–4 per scan	0.8–1.5 3.6–7.8 (ages 3–5) 1.3–1.8 (age 12)	1. Size-independent 2. Measures cortical and cancellous bone separately 3. Measures bone, muscle and fat	1. Skilled staff required for examination 2. Long scan time 3. Can only measure peripheral sites

The table is adapted from "Bone Densitometry in Growing Patients: Guidelines for Clinical Practice" (chapter 2) and Söderpalm et al. [98]. DXA=dual-energy X-ray absorptiometry, DXL=dual-energy X-ray absorptiometry and laser, pQCT=peripheral quantitative computed tomography.

1.14 Impact of mechanical loading on bone

In the earlier years of skeletal physiology, during the 1960s, it was thought that the effector cells in bone tissue (chondroblasts, fibroblasts, osteoblasts and osteoclasts etc.) alone determined the characteristics of bones, joints, fascia, ligaments and tendons. This was attributed to regulation from non-mechanical agents such as calcium, vitamin D, hormones, genes and age. This view was changed by Harold Frost and his mechanostat theory, which suggests that all skeletal organs (cancellous bone, cortical bone, growth plate, articular cartilage, tendons, ligaments and muscle) adapt their structure, strength and stiffness to the voluntary loading that is exerted on them. There are three states bone can have: disuse, adapted and overload which finally results in fracture. Between the disuse and adapted state and between adapted and overload there are threshold ranges for strain (bone deformation during loading). During the disuse state there is bone loss; in the adapted state, bone mass is held in steady state; and in the overload state, modelling occurs, that is to say, bone mass is increased [105]. This theory has been examined in intervention studies, with reports of a positive effect of physical activity on bone quality in children [106-108].

1.15 WBV training

The modern form of WBV was a further development of, rhythmic neuromuscular stimulation, developed by the Soviet scientist Vladimir Nazarov [109]. In 1960, Bierman [110] conducted a case-control study with handheld cycloid vibration equipment, in which paravertebral muscles in the lower back and posterior aspects of lower extremities were stimulated. He could demonstrate that trunk flexibility increased in the vibration group, which he attributed to muscle relaxation. In a well-known study by Rubin et al. [111], the hind limbs of sheep were stimulated with vibration therapy (30 Hz, 20 minutes per day, five times per week for one year) and showed a twofold increase in bone formation, a 30% increase in cancellous bone volume and a 34% increase in cancellous bone density. Vibration therapy with the Galileo 2000 vibration training device (Novotec, Pforzheim, Germany) (Fig 8) has now been incorporated in the current space programme, where the aim is to prevent muscle and bone loss in astronauts [112].

Figure 8. A co-researcher standing in foot position 2 on a Galileo Med Basic WBV machine.

There are two principal WBV techniques in the commercially available devices: the vertical technique, in which vibration is transferred to both feet synchronously, and the side-alternating technique, in which the right foot is synchronously lowest when the left foot is highest, and vice versa [113]. The side-alternating technique is more analogous to walking and running, and may be associated with less risk of negative side effects [114].

During the last decade, WBV training has gained in popularity and nowadays a WBV device can be found in every larger fitness centre. With the ongoing popularity of these devices, an increasing number of studies have been performed and a guideline document on how to conduct and report studies has been published [115].

WBV has been introduced as an alternative or supplement to regular physical activity and as a treatment option for several clinical conditions associated with loss of musculoskeletal mass, including osteoporosis and muscle strength [116-118]. It has also been evaluated as a treatment option for improving mobility in severely motor-impaired children and adolescents [104, 119-121]. It has been demonstrated that WBV training has a positive effect on fasting blood glucose [122-125], insulin [122], HbA1c [123, 126], weight [127], bone formation and resorption [128-130].

Nonetheless, there is a limited number of studies on WBV training in children, and specifically in children with obesity. The only study performed in young children with obesity was by Erceg et al. [131] who investigated the effects of WBV on bone mass, glucose and bone metabolism in 10-year-old overweight boys. Erceg et al. [131] could only demonstrate a positive effect of WBV on BMC and BMD.

2 AIMS AND HYPOTHESES

General aim

- The general aim of the thesis was to investigate the interplay between bone and fat tissue, with focus on OC, through clinical studies in children and adolescents.

Specific aims.

- To clarify the role of OC in relation to weight, with focus on ucOC and cOC, in children and adolescents (papers I–IV).
- To gain insight into how obesity and underweight affect bone and fat tissue in children and adolescents, with focus on ucOC and cOC (papers II–IV).
- To explore the relationship between weight, anthropometric data and bone mass (papers I, III and IV).
- To study the effect of WBV on energy and bone metabolism, anthropometric measurements, muscle parameters and BMD (paper IV).

Hypotheses

- OC, and specifically ucOC, is associated with a favourable metabolic profile in children and adolescents (papers I–IV).
- Weight has a positive effect on BMD (paper I and paper III).
- Adiponectin is inversely associated with bone mass in children and adolescents (paper I and paper III).
- WBV training increases muscle strength, which in turn improves the metabolic profile and BMD (paper IV).

3 PATIENTS AND METHODS

3.1 Study Subjects

Paper I

Subjects in paper I were included from the Gothenburg cohort (1825 children between two and nine years of age) in the IDEFICS study and were examined during the baseline survey (n=1825). All children in the age range 4.0 to 5.0 years were selected. This gave a total of 41children (28 boys) in this sub-study. Each of these 41 subjects underwent a heel DXL scan. Twenty-nine subjects also had blood samples taken during the IDEFICS examinations. The group was divided into normal-weight and overweight/obese according to BMI standard deviation score (BMI-SDS) [132]. Because the IDEFICS examinations were performed several months before the heel DXL, we also measured height and weight at the time of the heel DXL examination. The correlation between these two measurements was r=0.79 (P=0.001).

Paper II

In paper II the subjects were also included from the Gothenburg cohort in the IDEFICS study. The 62 children who were the most overweight or obese and had provided a sufficient blood sample were included, as well as 46 normal weight children. The inclusion criterion was overweight or obesity according to Cole et al. [133]. The children were matched for age (maximum difference of six months), gender and the month when the blood samples were taken. All included subjects were between two and nine years of age.

Paper III

Twenty-seven participants met the eligibility criteria and were asked to participate during the inclusion period (January 2012 to July 2014). Inclusion criteria for the study were age between 16 and 24 years and diagnosis of AN according to the DSM IV [134]. Exclusion criteria were diabetes mellitus, inflammatory bowel disease or any physical condition that demanded care at an internal medicine department. Twenty-five patients agreed to participate of whom three did not complete the study: two because they did not want to go through the 12-week programme and one due to reconsideration of the AN diagnosis. Thus, 22 patients completed the study according to protocol.

Paper IV

Children referred to the Queen Silvia Children's Hospital (Gothenburg, Sweden) were recruited to the study. A total of 19 girls and 17 boys, from seven to 17 years of age (median 13 years) were included. Patients were randomized into the WBV group (n=19) or the control group (n=17). The website https://www.sealedenvelope.com/ was used for the randomization procedure, which was performed in blocks with a total of four possibilities, divided into two for the WBV group and two for the control group, based on age and gender categories. The following age categories were used: 7–10, 11–13 and 14–17 years, divided by gender. Within each of these six categories, there was a maximum of 10 envelopes to be chosen from each pile. In total there were 60 envelopes containing the option WBV or control. The randomization block was open to one of two researchers who selected the subjects. Three subjects dropped out from the WBV group: one declined after reconsideration (before study start), the second because of social and family problems and the third because of headaches. The control group consisted initially of 17 subjects, but one dropped out immediately after randomization because she was not randomized to the WBV intervention group, and two subjects declined to attend the study examinations at week 12. A total of 14 girls and 16 boys completed the entire study period. The final WBV group comprised 10 girls and six boys in the following age categories: 7–10 (n=4), 11–13 (n=5), and 14–17 (n=7) years. The final control group comprised eight girls and six boys in the following age categories: 7–10 (n=3), 11–13 (n=4), and 14–17 (n=7) years.

The inclusion criterion was obesity defined as BMI-SDS >2 according to Karlberg et al. [132]. Exclusion criteria were serious attention deficit hyperactivity disorder on pharmacologic treatment, severe syndrome diagnosis, chronic diseases with regular medication (e.g., diabetes mellitus and juvenile idiopathic rheumatoid arthritis), children from rehabilitation clinics or with severe communication difficulties (including low language proficiency).

3.2 Study designs

In paper I and paper II the study design was cross-sectional. In paper III the study design was longitudinal and in paper IV the study design was randomized, controlled and longitudinal.

3.3 Osteocalcin – analytical methods

The principal difference between methods to analyse OC is that they use two different techniques: radio-labelled assays and enzyme-labelled assays. The most common methods at present are enzyme-labelled assays, for example enzyme-linked immunosorbent assay (ELISA), also known as enzyme immunoassay (EIA) and electrochemiluminescence immunoassay (ECLIA). Osteocalcin is a protein containing 49 amino acids [135] and in serum different fragments can be found in serum (Fig 9) When choosing which assay to use, it is important to take into account which OC fragments the assay can detect; an assay that detects only intact OC will be sensitive to *in vitro* degradation, whereas assays detecting fragments can overestimate the concentration of intact OC [136].

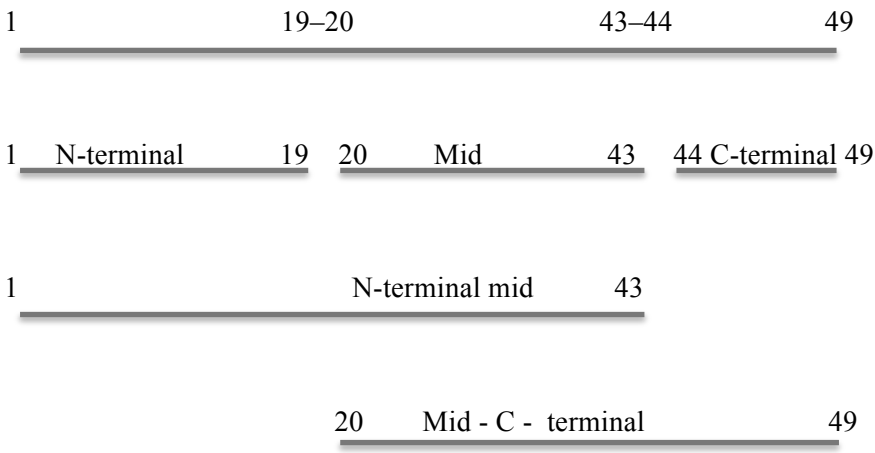

Figure 9. Circulating immunoreactive forms of OC, intact and fragments, as reported by Garnero et al. [137].

ELISA

In brief, an antigen is attached to a surface and a specific antibody is applied over the surface, which binds the antigen. An enzyme is linked (conjugated) to this antibody, and finally a substrate is added and converted by the enzyme into a signal, such as a colour change in a chemical substrate. This colour has a specific wavelength and it can be converted to a specific concentration

[138]. In **paper I**, the ELISA from Immunodiagnostic Systems Holdings PLC (IDS), Boldon, UK was used to analyse total OC, which measures intact OC (amino acids 1–49) and the N-terminal mid-OC fragment (amino acids 1–43).

Radioimmunoassay

Radioimmunoassay can be used to determine analytes (e.g., hormones and proteins) at low concentrations in serum. The method requires an antibody specific to the molecule to be measured and a radiolabelled version of the molecule that contains a soluble radioisotope, for example radioactive iodine. A known quantity of the radiolabelled molecule appears in the solution. The patient's serum is added to the solution with the antibody and the radiolabelled molecule. The unlabelled molecule in the patient's serum competes with the radiolabelled molecule to bind to the antibody. The higher the concentration of the specific molecule in the patient's serum, the more of the radioactive molecule will be displaced. The antibody-bound molecule is then separated from the free molecule and the radioactivity of the bound molecule remaining in the supernatant can be measured with a radioactive counter, e.g. a gamma counter [139].

ECLIA

Electrochemiluminescence immunoassay (ECLIA), or electrogenerated chemiluminescence, is luminescence that is produced during electrochemical reactions in a solution. The patient's serum is incubated with two antibodies. The first antibody is labelled with biotin and the second with ruthenium; both substances are specific for the antigen in serum and bind to it like a sandwich. Thereafter, streptavidin is introduced in the solution and binds to biotin, after which the immunoacid complex is transferred to the measuring cell. Here, a magnet is applied, tripropylamine is introduced and an electrogenerated chemiluminescence reaction is started when voltage is applied. The light is emitted when electrochemically generated intermediates such as ruthenium pass from an excited state to a relaxed lower-level state. The signal detected is equivalent to the concentration of the target analyte (Fig 10). Advantages of the ECLIA method are rapid measurement, a wide measuring range, a controlled reaction, precision, sensitivity and low sample volume [140]. In **paper II** and **IV** ECLIA methods were used to analyse total OC. In **paper III**, the CLIA method (also a chemiluminescence technology) from Diasorin was used. The method is based on the same principles as ECLIA.

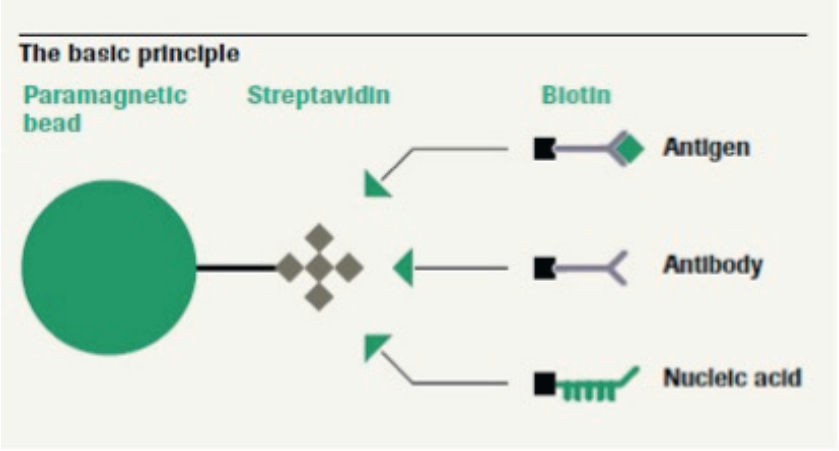

Figure 10. The streptavidin-biotin bond is used to affix the antigen-antibody complex to a paramagnetic microbead. Different immunoassay types are feasible for example, competitive and sandwich. Paramagnetic microbeads enable a controlled capture and release of the antigen-antibody complex when magnetic forces are applied[140]. © Roche Diagnostics Limited.

Table 4. Assays for detecting serum OC.

Assay	Supplier	Method	Fragments detected	Volume	Sample	Intra-assay CV (%)	Inter-assay CV (%)	Limit of detection
N-MID® Osteocalcin ELISA	IDS PLC.	ELISA	Intact (1-49) N-terminal mid fragment (1–43)	20 µL	S, P	4.5	3.5	0–100 ng/mL
Human Gla-OC High Sensitive EIA Kit	Clontech Takara	ELISA	Gamma-carboxyglutamate at position 17. (undercarboxylated OC)	-	S	2–3.4	4.2–6.2	0–12 ng/mL
N-MID® Osteocalcin ELISA	IDS PLC.	ELISA	Intact (1-49) N-terminal mid fragment (1-43)	20 µL	S, P	4.5	3.5	0-100 ng/mL
Human Gla-OC High Sensitive EIA Kit	Clontech Takara	ELISA	Gamma-carboxyglutamate at position 17. (carboxylated OC)	-	S	2 - 3.4	4.2 – 6.2	0.2-12 ng/mL
Undercarboxylated Osteocalcin (Glu-OC) EIA Kit	Clontech Takara	ELISA	Undercarboxylated OC	220 µL	S, P	4.58 - 6.66	5.67 – 9.87	0.25-8 ng/mL
Osteocalcin (bone GLA protein) immunoradiometric assay kit	Cisbio Assays	IRMA	Intact OC (1-49) Human OC peptide (1–43)	50 µL	S, P	1.2–2.8	3.6–5.2	0–300 ng/mL
LIAISON®Osteocalcin	Diasorin	CLIA	N-terminal mid fragment (1–43)	225 µL	S	3–8	4–9	0.3–300 ng/mL
ROCHE	Roche	ECLIA	N-terminal mid fragment (1–43)	-	S, P	0.9–1.3	1.4–3.3	0.5–300 ng/mL

S=serum, P=plasma, ELISA=enzyme-linked immunosorbent assay, CLIA=one-step sandwich chemiluminescence immunoassay, IRMA=Immunoradiometric assay.
Manufacturers: http://www.idsplc.com/products/n-mid-osteocalcin-elisa/,

http://www.clontech.com/SE/Products/Cell_Biology_and_Epigenetics/Bone_Research/Osteocalcin_Human?sitex=10120:22372:US,

http://www.cisbio.com/other/diagnostics/osteocalcin-bone-gla-protein-immunoradiometric-assay-kit-protocol,

http://catalog.diasorin.com/en/ricerca/1,3,7281,,CLIA,

https://usdiagnostics.roche.com/products/12149133160/PARAM328/overlay.html

3.4 Bone measurements

Heel DXL

The DXL Calscan system Calscan (Demetech AB, Täby, Sweden) was used to measure left calcaneal BMC and BMD in subjects. The interoperator CV for BMD was 5.3%[100]. The DXL Calscan was used to measure bone mass in papers I, III and IV.

DXA

In **paper III**, BMD and BMC were measured with DXA (Lunar Prodigy, GE Lunar Corp., Madison, WI, USA) for total hip, total body, lumbar spine (L1–L4), left arm and left leg. Age- and gender-specific Z-scores were calculated automatically. Fat mass and lean body mass were also assessed. In order to assess the *in vivo* precision, 20 healthy subjects (aged 6–37 years) were scanned twice by the same examiner. The calculated CV was 0.5% for total body BMD and 0.7% for spine (L1–L4) BMD, 2.4% for lean mass and 0.9% for body fat mass [141].

Peripheral QCT

In **paper III**, pQCT measurements were performed on the left tibia at 4% and 66% of the tibia length using an XCT 2000 (Stratec Medizintechnik GmbH, Pforzheim, Germany) with software version 6.00. A quality control calibration was performed before each measurement. The tibial length was measured from the medial malleolus to the medial tibial plateau. The exact position of the CT scans was defined in a coronal scout scan at the foot ankle joint. With the scout scan as reference, the tibial 4% and 66% were determined. From these regions, tibial and cortical volumetric BMD, cortical thickness, polar SSI (mm3), cortical cross-sectional area, periosteal and endosteal circumference were calculated. The performance of the device has been reported elsewhere [142]. All pQCT measurements were carried out by the same staff. Ten individuals were measured by pQCT and the calculated intra-individual CV was 1.3% for total area, 0.8% for trabecular density and 0.4% for cortical density.

3.5 WBV training

Subjects performed WBV in their home environment on a Galileo Med Basic (Novotec Medical GmbH, Pforzheim, Germany) side-alternating vibration plate, which has been described in detail elsewhere by Semler et al. [120]. This WBV device ensures consistent targeting amplitude oscillating along the sagittal axis, which induces side-alternating oscillations that provoke apical gait-like movements of the whole body. Parents and subjects were instructed how to use the WBV device and received supervised training. Each foot was placed in a position that yields a vibration amplitude of 2 mm, which corresponds to a peak-to-peak displacement of 4 mm. WBV was performed at 16–24 Hz three times per week for 12 weeks in a 90°–130° squatting position with both feet aligned in marked positions, with the possibility to place the back of a chair in front of them for balance support. The basic WBV session started with a one-minute warm-up followed by one minute's rest, followed by three two-minute repetitions with one rest in between. During week 9 to week 12 the subjects were allowed to individually adjust the time per repetition to a maximum of three minutes per repetition. The frequency and amplitude was gradually enhanced from16 Hz to 24 Hz, according to how well the subject managed the training. The amplitude started at position 1 and the subjects could move to positions 2 and 3 as they felt stronger and more confident. Hence, all subjects had the same WBV programme at start, but they were free to adjust the frequency and amplitude within the programme. This WBV protocol was decided upon based on earlier studies [126, 131, 143, 144]. When vibration training was performed at 16–18 Hz, a peak acceleration of 2.1–2.6 g was transmitted to the body, and at 20–24 Hz the peak acceleration was 3.2–4.6 g. The WBV device recorded each training session to facilitate evaluation of the specific amount and intensity of WBV for each individual. Each family was contacted by telephone during the 12-week study period to identify possible problems and to monitor adherence.

3.6 Leonardo mechanography

Leonardo Mechanography® Ground Reaction Force Plate (GRFP) (Novotec Medical GmbH) was used to evaluate muscle parameters (Fig 11). The platform consists of two symmetrical separate force plates that are fitted in a wooden frame. The platform has eight force sensors, which measure the vertical ground reaction that is applied to it. The signal from the force plate is analysed with the software Leonardo Mechanography version 4.2 (Novotec Medical) [145]. Leonardo GRFP has been used in previous studies to evaluate muscle function in children and adults[146, 147]. Acquired

measurements for acceleration, velocity, muscular force, power output and jump height, were calculated using the GRFP software[148]. Muscle measurements were performed at baseline and at study week 12. All children were evaluated with the measurements single two-leg jump (S2LJ), multiple one-leg hopping (M1LH), chair rising test (CRT) and balance test (BT). The single two-leg jump was performed as a counter-movement jump ("natural jump") with freely moving arms, and children were instructed to jump as high as possible and to land on both feet. All subjects performed three jumps and the highest jump was selected. The outcome parameters were jump height (m), the Esslinger Fitness Index (EFI%) and the EFI-SDS. In M1LH, children were instructed to jump as fast as possible on one forefoot, with the knee stiff. The purpose was to achieve maximum voluntary forefoot ground reaction force during landing. A possible application of this test is to evaluate the maximal force to which the tibia is exposed, and thus evaluate the muscle–bone unit [146]. The outcome parameter was relative maximum force ($F.max_{rel}$), i.e. F.max in relation to body weight in grams.

Chair rising test was performed with a customized chair fixed to the Leonardo plate. Subjects were instructed to stand up to full knee extension and sit down again as fast as possible, five times in a row with both arms crossed over the chest. The maximal total relative power per body weight during the rise phases of the five chair stands ($CRTP_{rel}$) was used for analysis. During BT, children were instructed to perform a single-leg stance stand for 10 seconds, performed with both eyes open and then with both eyes closed. The objective was to evaluate balance, proprioception and coordination and the outcome parameter was Sway Index/Standard Ellipse Area (cm^2) as a measurement of the amount of swaying in relation to 90% of an age-specific standard ellipse (correlated to the coefficient of variation (CV)).

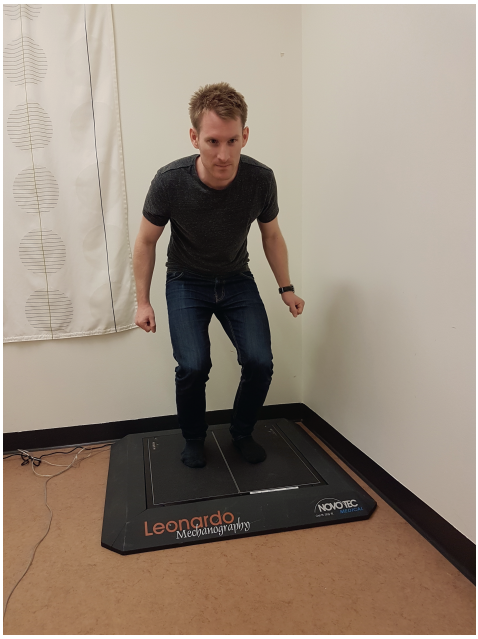

Figure 11. Co-worker Rickard performing the single two-leg jump on Leonardo.

3.7 Questionnaires

In **paper IV**, subjects and their parents had to fill out questionnaires at study start and study week 12. The questions at study start concerned training, diet, balance, current diseases and medicine and/or vitamin intake. The questionnaire at study week 12 covered vibration training, current fitness status, current general training level, diet treatment and general experience of the study. The parents answered questions regarding their experience of the WBV and the general experience of the study.

3.8 Statistical analysis

In **paper I**, values are given as mean, standard deviation (SD) and median. Anthropometric characteristics as well as bone density data and biochemical marker data were compared between the groups with the non-parametric Mann–Whitney U-test. Correlation between bone mass measurements and biochemical markers and anthropometric measures was performed with the Kendall's tau-b correlation coefficient. All tests were two-tailed and conducted at the 0.05 or 0.01 significance level. The software used was SPSS version 16.0 (SPSS Inc., Chicago, IL, USA).

Due to the many cross-correlations, a multiple regression analysis was performed to study determinants for the DXL bone mass parameters. The final model consisted of waist circumference, adiponectin, homeostatic model assessment (HOMA), calcium and CTX, and was run one by one for each of the three bone mass measurements (dependent variables – BMC, BMD, BMAD). Twenty-seven children were included in the models due to missing values in the explanatory variables. All models were adjusted for age and sex. A P-value of < 0.05 was considered as statistically significant. The software used was SAS version 9.2 (SAS Institute, Cary, NC, USA).

In **paper II**, values are given as mean and standard error of the mean (SE), median, minimum and maximum. Anthropometric and biochemical metabolic data were compared between the groups using the non-parametric Mann–Whitney U-test. All test were two-tailed and conducted at the 0.05 or 0.01 significance level. Correlations between the different forms of OC and the metabolic and anthropometric measurements were calculated with the Spearman's rank correlation coefficient, rho, at the 0.05 significance level. Corrections for multiple testing were performed with the Bonferroni correction and Holm's sequential Bonferroni procedure. A power calculation was performed with reference to two different studies on OC in children [149, 150]. To detect a difference in OC of 5.4 ng/mL (with alpha=0.05 and beta=0.10) and to detect a difference of 1.7 µg/mL (with alpha=0.05 and beta=0.20) in adiponectin, a total of 82 subjects were needed. The software used was SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA).

In **paper III**, dichotomous variables were expressed as number and percentage and continuous variables as mean, SD, median, minimum and maximum. To test changes in biochemical parameters, bone measurements and body composition over time within a group, the Wilcoxon signed-rank test was used. To assess the correlation between the different OC forms and biochemical parameters, bone and body composition measurements, Spearman's rank-order coefficient, r_s, was used. All tests were two-tailed and conducted at the 0.05 significance level. All analyses were performed with SAS Software version 9.3 (SAS Institute, Inc., Cary, NC, USA).

In **paper IV**, dichotomous variables were described by numbers and percentage values, and continuous variables by mean, SD, median, minimum and maximum. To test changes in anthropometric characteristics, biochemical markers, muscle strength and heel bone mass parameters within groups, the Wilcoxon signed-rank test was used. When comparing the groups for the previously mentioned parameters, the non-parametric Mann–Whitney U-test was used. All tests were two-tailed and conducted at the 0.05

significance level. All analyses were performed by using the IBM SPSS Statistics for Macintosh, Version 23.0 (IBM Corp., Armonk, NY, USA).

3.9 Ethics

All studies were approved by the regional ethics committee at the University of Gothenburg (approval number 264-07 for **paper I** and **paper II**, 720-11 for **paper III** and 387-13 for **paper IV**).

4 SUMMARY OF PAPERS / RESULTS

Paper I

Introduction

The aim of this pilot study was to investigate the relationship between bone and proxy variables for growth and glucose metabolism.

Subjects

Forty-one overweight/obese (n=13) and normal-weight (n=28) subjects (14 girls) were chosen from the IDEFICS study´s Gothenburg cohort of 1825 children.

Methods

In all 41 subjects, heel DXL was performed, and 29 subjects gave blood samples. Anthropometric measurements such as height, weight, skinfold and waist circumference were recorded for all 41 subjects.

Results

The bone mass measurements BMC, BMD and BMAD differed between overweight and normal-weight children. BMI-SDS correlated positively with all three measurements: BMC ($r=0.36$, $P<0.01$), BMD ($r=0.34$, $P<0.01$), and BMAD ($r=0.29$, $P<0.01$). Waist circumference also correlated positively with all three measurements: BMC ($r=0.32$, $P<0.01$), BMD ($r=0.30$, $P<0.01$) and BMAD ($r=0.26$, $P<0.05$). While subscapular skinfold correlated positively with all three: BMC ($r=0.26$, $P<0.05$), BMD ($r=0.25$, $P<0.05$) and BMAD ($r=0.23$, $P<0.05$), the sum of skinfold measures only correlated significantly with BMC ($r=0.25$ $P<0.05$) and BMD ($r=0.23$, $P<0.05$). Adiponectin was significantly inversely correlated with all three measurements: BMC ($r= -0.41$, $P<0.01$), BMD ($r= -0.40$, $P<0.01$) and BMAD ($r= -0.41$, $P<0.01$). In a multiple regression analysis, adiponectin was an independent determinant of all three bone mass measurements. Osteocalcin was not correlated with any of the measured parameters.

Discussion

The differences between overweight and normal-weight children were least significant regarding BMAD, which could be explained by the correction for bone size that was made by measuring calcaneal height when calculating BMAD. The inverse correlation between adiponectin and bone mass measurements could be mediated via Wnt signalling inhibitor Dickkopf-1 (DKK-1) by inhibition of osteoblastic differentiation [151]. The study was conducted in very young subjects, which is a strength of the study. Limiting factors in the study are the small sample size and the fact that the blood sampling and heel DXL measurements were collected at different time points.

Conclusion

The negative correlation of adiponectin with BMC, BMD and BMAD has previously not been shown in very young children. Adiponectin may have an independent role in bone development and metabolism in young children.

Paper II

Introduction

Osteocalcin is suggested to be inversely associated with parameters of glucose metabolism. Earlier studies have primarily been performed in human adults and in rodents. The assumed interplay between bone tissue and energy metabolism have not been investigated in young children. In this paper we aimed to clarify the possible association between OC, metabolic parameters and anthropometric characteristics in normal-weight and overweight/obese children.

Subjects

The study included108 Swedish children (46 normal weight, 62 overweight/obese) aged two to nine years.

Methods

Anthropometric data (height, weight, skinfold, waist circumference, insulin, glucose, glycosylated haemoglobin (HbA1c), HOMA index, vitamin D, adiponectin, total OC, cOC and ucOC were analysed.

Results

Total OC and ucOC did not differ between the normal-weight and overweight/obese groups, but cOC levels were lower in overweight/obese children, mean 69.1 (±2.2) ng/mL, than in normal-weight children, mean 75.6 (±2.5) ng/mL (P=0.03). None of the three OC forms correlated with any of the measured anthropometric or metabolic parameters after correction for multiple comparisons.

Discussion

A possible explanation for the observed differences between mice and humans could be the different number of OC genes in the respective species. The assays for measuring the different OC forms are also suboptimal, which complicates inter-study comparisons. If the two groups had differed even more regarding weight status, it would probably have been easier to detect differences in OC levels. The lower level of cOC in overweight children gives reason to further focus on exploring cOC as potentially favourable in glucose metabolism. The assessment of all three OC forms (total OC, ucOC and cOC) in very young subjects is a strength of this study. Limiting factors are the low sample size, the cross-sectional design and the fact that no other bone turnover markers were measured.

Conclusion

Levels of cOC were lower in overweight prepubertal children in comparison with normal-weight children; however, the study groups did not differ with respect to ucOC. The three OC forms were not associated with any of the measured anthropometric or metabolic parameters. Thus, this study does not support the hypothesis of an association between OC and a positive metabolic profile.

Paper III

Introduction

Patients with anorexia nervosa (AN) are at high risk of reduced bone mass. The aim of the study was to investigate how total OC, ucOC and cOC respond during a 12-week intensive nutrition therapy in AN patients, in contrast to **papers I**, **II** and **IV**, where the focus was on overweight/obese subjects.

Subjects

Twenty-two female AN patients, mean age 20.9 years, with a starting mean BMI of 15.5 kg/m^2 participated in the study.

Methods

Blood samples were collected, body composition and bone mass were assessed by DXA and pQCT, and a heel DXL was performed.

Results

Subjects gained a median of 9.9 kg in weight and BMI increased from a median of 15.4 kg/m^2 to 19.0 kg/m^2, P<0.0001. Fat mass increased from median 11.4% to 26.7%. Total OC, cOC, ucOC and bone ALP increased. The resorption marker CTX did not change. Total body BMC increased, but no changes were found for whole body or lumbar spine BMD. Tibial trabecular density measured by pQCT decreased. Total OC, cOC, and ucOC did not correlate with BMI, insulin or body composition parameters.

Discussion

The median weight gain of 9.9 kg supports the effectiveness of the treatment regime with a high-energy intake directly from start. This study investigated all three forms of OC, which previous studies of weight gain treatment in AN patients have not assessed [152], [153]. The increase in total OC and bone ALP, along with the unchanged CTX levels indicate a positive net gain in each remodelling cycle, which may explain the increased BMC values. The rise in all three OC forms could be explained by the sudden increase in nutrition as the intense high-calorie diet was initiated, which in turn activated bone formation through osteoblasts, resulting in increased OC synthesis [154]. The lack of a control group is a limitation in this study.

Conclusion

In this paper we demonstrated that all three forms of OC (total OC, cOC and ucOC) increase during rapid weight gain, although the absence of a correlation with insulin and anthropometry changes meant that we were unable to confirm earlier findings of a favourable role of OC for glucose metabolism. The increase in bone ALP and unchanged levels of CTX corroborated the increased total body BMC.

Paper IV

Introduction

There is a need for new approaches in the endeavour to increase physical activity and improve metabolic profile in obese children. The aims of this paper were to study the effect of WBV in these children on: (i) biochemical markers of energy and bone metabolism, (ii) anthropometric measurements, (iii) muscle parameters and (iv) calcaneal BMD.

Subjects

Thirty-six obese children, aged 7–17 years, were randomized to WBV or a control group in a prospective controlled study.

Methods

WBV was performed at 16–24 Hz three times per week for 12 weeks in a home setting, and study parameters (anthropometric data, blood samples, lower extremity muscle strength measurements, heel DXL and questionnaires) were assessed at start and after 12 weeks.

Results

Thirty children completed the study, and adherence was 51% of the expected WBV time. Sclerostin, bone ALP and CTX decreased in the WBV group ($P<0.05$), but OC and insulin remained unchanged. Balance improved in the WBV group ($P<0.006$). Anthropometric data, muscle strength and calcaneal BMD did not differ between the two groups.

Discussion

There are few randomized controlled studies of WBV in obese children, which makes the current study unique. Due to the low adherence for performing the WBV training and the low number of subjects, the likelihood of significant positive results was limited. The WBV was regarded by some of the subjects as monotonous, and adherence would probably have been better if the WBV intervention had been more varied. This home-based WBV intervention programme without proper surveillance cannot be recommended. WBV training could be a way to motivate children with a low degree of physical activity. The low adherence to performing WBV could explain the absence of effects on bone mass measurements in contrast to

earlier studies [119, 121, 131]. The decrease in sclerostin in the WBV group confirms the role of sclerostin as an early marker for muscle stimulation of bone through the WBV device.

Conclusion

This WBV intervention study improved balance but did not affect metabolic parameters, anthropometric data or muscle strength. The reduction of sclerostin implies that WBV therapy in obese children has direct effects on osteocytes, the key players in bone mechanotransduction.

5 DISCUSSION

Clarifying the role of OC, with focus on ucOC and cOC.

As mentioned in the Introduction, it has been proposed from mice studies that OC, specifically ucOC, counteracts obesity, hyperglycaemia and insulin resistance [52, 53, 155]. Validation of these findings in studies on humans has produced diverging results (Table 2). Most human studies have analysed total OC. One of the aims of this thesis was to further explore the possible role of total OC, as well as ucOC and cOC, in relation to weight, insulin and glucose levels in children and adolescents.

The number of studies focusing on the interplay between OC and measures of adiposity in children are few and the results have been divergent. One group of studies demonstrated favourable associations between total OC, ucOC or cOC and measures of glucose and insulin homeostasis, such as HOMA index [71, 156], glucose [157, 158], insulin [75] or HbA1c [156-158]. But other studies have not demonstrated any association between total OC, ucOC or cOC and HOMA [70, 72, 159], glucose [70, 159], insulin [70, 71, 75, 159] or HbA1c [160]. In **paper I**, we could not demonstrate any association between total OC and indices of weight, such as BMI-SDS or HOMA index, as a marker of glucose and insulin metabolism. A potential explanation for the lack of confirming findings in **paper I** is the low number of subjects included.

Early research in this field focused on OC as a potential regulator of glucose metabolism and fat mass in rodent studies, in particular the undercarboxylated form of OC. It was suggested that ucOC was the metabolically favourable form [52-54, 155]. With this background we aimed to explore the role of ucOC in young children and adolescents. At the time when the study leading to **paper II** was planned and performed, there had not been many publications exploring total OC, ucOC and cOC in humans in general and even fewer specifically in children. A majority of the studies mentioned have solely analysed total OC (Table 2), which is a limitation in the design of these studies that makes it difficult to compare the results with the mouse studies, in which the focus has been on ucOC and its favourable effect on the metabolic profile. These circumstances were partly due to a lack of reliable assays and methods to measure the three different OC forms. Boucher-Berry et al. [71] reported an inverse relationship between ucOC and HOMA index in 106 children (mean age 12.6 years). Pollock et al. [75] performed a study in obese prepubertal children (mean age nine years)

divided into a prediabetic and normal glucose group. They reported a positive association between total OC and cOC with an insulin sensitivity index (Matsuda index) and an inverse association between cOC and fasting insulin. Furthermore they could demonstrate a positive association between ucOC and the insulinogenic index (as a marker of β-cell function) as well as a decreased level of ucOC in the prediabetic group.

In **paper II**, markedly more subjects were included, and we explored whether there was any association between the three different OC forms and glucose, insulin, HOMA index and anthropometric measurements such as waist circumference. After adjusting for multiple comparisons, we could not find any of the hypothesized associations. These results are in contrast to the earlier findings described above. In **paper III**, we intended to explore the other end of the weight spectrum, in our case a study of patients with AN gaining weight over a period of three months. We expected a positive correlation between insulin levels and levels of total OC, ucOC and cOC, which we could not demonstrate.

Concerning the relationship between OC and weight status represented as BMI or BMI-SDS, the results diverge: some studies report an inverse association [70, 71, 81, 156], while others do not demonstrate any correlation between OC and weight status [157, 161, 162]. A positive association has been demonstrated between cOC and weight (BMI) [79]. In **paper I**, we could not support any difference in levels of total OC between normal-weight and overweight four-year-old children. In **paper II**, the aim was to examine whether there is a difference in levels of total OC, as well as ucOC and cOC, between normal weight and overweight/obese children. We could only find a significantly increased level of cOC in normal weight children, but we found no correlation between BMI-SDS and any of the OC forms. These findings are in contrast to Viljakanen et al. [74], who detected higher levels of total OC and cOC in obese subjects after oral glucose tolerance tests. The diverging results in **paper II** could partly depend on the low average BMI Z-score of 1.8, which is just below the cut-off for obesity. In **paper III**, we could not demonstrate any association between weight increase and levels of the three different OC forms.

It is important to take into account different confounding factors, for example, diet, physical activity, or bone mass itself, when analysing the associations between OC and parameters related to adiposity [163]. **Paper I** and **paper II** have a cross-sectional design, which is why we cannot draw any conclusion about a temporal or causal relationship between OC levels and markers of glucose homeostasis and weight status (as measured by BMI,

BMI-SDS, or waist circumference). We did not measure vitamin K, which should be measured in future studies examining the relationship between OC and glucose homeostasis. Vitamin K could be a potential link between OC and glucose homeostasis [164]. There are also reports of increased OC levels in obese subjects [74, 165]. This could be explained by the suggestion that human adipose tissue produces and contains both ucOC and cOC protein, which could also mean a positive local impact of OC on adipose tissue [82]. There are no general criteria for deficiency in ucOC levels, which also makes it difficult to draw conclusions.

A factor that could partly explain the different findings between mice and humans is the fact that mice have three OC genes and humans have only one; this could indicate different regulative mechanisms and alternative pathways [166]. It could be that cOC in humans (also partly indicated in **paper II)** has a more prominent role than what was hypothesized from the beginning of research in this field[167] and that ucOC is not the main contributor, as stated by Yoshida et al. [168].

The results reported in this thesis do not support the hypothesis of an association between OC and a positive metabolic profile. It still remains unclear whether OC is only a BTM or also functions as a link between bone and glucose metabolism in humans. Longitudinal and large-scale studies are needed to further explore the precise roles of the different OC forms in relation to glucose homeostasis in children and adolescents of different ages and BMI status.

The focus on the research area of OC and its potentially positive effect on glucose and insulin metabolism came, as pointed out in the Introduction , after mice studies conducted almost solely by Karsenty and colleagues [52, 53, 155]. This is a limiting factor for the whole research field, as the results would be more reliable if reproduced by other independent groups.

In **paper IV**, one of the aims was to clarify whether WBV has an effect on BMD and whether levels of total OC, ucOC or cOC would increase in the WBV group. We did not find any effect on OC levels in the intervention group. This could be due to lack of patient adherence and low statistical power.

Methodological aspects of BTM

When discussing studies that compare BTM levels, it is important to take into account several sources of variability [169, 170], such as pre-analytical conditions, specificity of the assays used and the biological availability of the BTM in the blood [171]. Examples of important pre-analytical conditions are age, gender, pregnancy and lactation, menopausal status, drugs, disease, fracture and bed rest/immobility [169]. For most BTMs, a morning fasting sample is recommended and the circadian rhythm of the BTMs (especially bone resorption markers) should be taken into account [172]. Furthermore, intact OC is unstable *in vitro* and degrades fast in room temperature at 4°C [136, 173]. In the studies reported in this thesis, all of the samples were collected in the early morning (fasting) and were handled accordingly to the instructions, which meant that samples were centrifuged and placed in a freezer at −20°C within 2 hours from sampling, and after a period placed in long-term storage at −80°C. This minimizes the pre-analytical variation and degradation of the BTM and increases the reliability of the results reported in this thesis.

When analysing the results of different studies, it is important to be aware of the technical specification of the OC assay used and the epitope specificity for the monoclonal antibodies used, as well as whether intact OC and/or different fragments are detected. Assays detecting intact OC are extra sensitive for *in vitro* degradation, whereas there is a risk of overestimating intact OC concentration with assays that detect fragmented OC [136]. In **paper I**, the ELISA method from IDS was used to determine total OC (amino acids 1–49) and the N-mid fragment of OC (amino acids 1–43). In **paper II** and **paper IV**, the ECLIA method was used to determine total OC and the N-mid fragment of OC, and the HA method [174] was used to determine ucOC. In **paper III** the CLIA (resembles ECLIA) method from IDS was used to determine total OC and N-mid fragment while Takara ELISA kits were used to analyse ucOC and cOC. The fact that intact OC and the N-mid fragment were measured in all four papers presented in this thesis is valuable when drawing conclusions based on all of the studies. The use of the two different methods (for practical reasons) to determine ucOC and cOC is limiting because of their different approach in the assay method. Different assays measuring OC have been reported in the past, delivering discordant results when compared against each other [137, 175].

Adiponectin, fat and bone tissue

OC interacts with adiponectin, which has a regulative effect on bone mass and adipose tissue. It has been demonstrated that adiponectin and its receptors are expressed on human osteoblasts [176]. Adiponectin has been suggested to have a functional role in bone metabolism, linking adipose tissue to body weight and BMD [151, 176, 177]. Low adiponectin levels have been linked to overweight and inactivity [178, 179]. In **paper I** and **paper III**, we have confirmed the inverse correlation between adiponectin and BMD that has been demonstrated in AN patients [72, 180], normal weight subjects [178] and obese subjects [181]. In **paper I**, we suggested that adiponectin could have an independent inverse association with BMD, BMAD and BMC in a multiple regression model with HOMA, β-CTX, calcium and waist circumference. This independent association of adiponectin with bone mass measurements has also been proposed earlier [181]. It could be speculated that adiponectin in some way has a negative regulatory effect on bone mass, taking into account the starkly diverging concentration levels of adiponectin in obese versus underweight subjects. This negative regulatory effect could be mediated through the Wnt-signalling inhibitor DKK-1 [151, 182].

In **paper I** and **paper II**, adiponectin was inversely associated with BMI-SDS and with BMI; this was not the case in **paper III** or **paper IV**, where we could not demonstrate any association between weight status and adiponectin. Levels of adiponectin are lower than normal in obese subjects [85, 183, 184] but higher than normal in AN subjects [12, 179]. However, in **paper I** and **paper II** we did not find the expected lower levels of adiponectin in obese subjects compared to normal-weight subjects. An explanation for the absence of a difference could be the very young age of the subjects (mean 4.5 years in **paper I** and 6.8 years in **paper II**). In earlier studies of adiponectin, an inverse association between adiponectin and weight status (BMI, BMI-SDS) was reported [185, 186].

Adiponectin was not associated with OC in any of the four studies reported in the current thesis. One possible explanation for the lack of association is that we did not measure high-molecular-weight adiponectin, which is suggested to have the highest biological activity (in comparison to low-molecular-weight adiponectin) with regard to the control of glucose homeostasis and fatty acid oxidation [187, 188].

To explore the interplay between weight increase and BMD

In **paper I** we demonstrated a difference between normal-weight and overweight subjects regarding bone mass measurements in the calcaneus and also a positive correlation between BMI-SDS and BMC, BMD and BMAD. We measured calcaneal height with heel DXL (paediatric version), which produces the parameter BMAD, and this parameter showed the least significant difference between the two groups. However, the variables were not adjusted for weight. Söderpalm et al. [100], also demonstrated a positive correlation between BMI-SDS and BMD in a study with a pooled group of children aged two to seven years. Obesity has been shown to have a positive impact on bone mass in children [189], but other studies indicate that the effect of obesity on bone mass parameters such as BMD and BMC disappears when adjusted for body weight, fat mass or lean mass [190, 191].

In **paper III** there were no improvements in BMD in AN patients during the 12-week study period, despite a mean 10 kg increase in weight, which confirms earlier findings [192]. Total body BMC did increase, a result that has been demonstrated by others [152]. Tibial cancellous bone density measured by pQCT decreased, while there was no effect on tibial cortical density. Calcaneal BMD and BMC also decreased. There are several possible explanations for the lack of effect on cortical bone. Firstly, the surface-to-volume ratio is about four times higher in typical cancellous bone than in cortical bone [193]. Secondly, bone is a heterogeneous organ with different ratios of cancellous and cortical bone throughout the body, and site-specific differences in the remodelling rate could all explain measurement heterogeneity at various bone sites. Finally, the short study period of 12 weeks is not long enough for results to appear, given the long remodelling cycle of cortical bone.

Generally when analysing DXA results, it is important to take into account the limited ability of the software to discriminate newly acquired adipose tissue from newly formed bone tissue [194]. In **paper III**, bone area and BMC had increased without a concomitant change in BMD; these results can be explained by weight changes without a true change in BMD, which has been suggested earlier [195].

The observation that AN patients display decreased or arrested growth and also demonstrate low bone mass when they are adults lends support to the theory that the skeleton has high energy demands [51]. In contrast, obesity has in the past been associated with high mechanical loading and increased BMD, although in recent studies this theory was contradicted when

researchers found a negative association between fat mass and BMD [52]. What seems primarily important is the amount of lean mass that the individual has [52, 53]. The main predictor for higher BMD and BMC values is suggested to be fat-free mass as a proxy for lean mass [196], which can explain the lack of increase in BMD (**in paper III**) because lean mass, as expected, did not increase.

Effect of whole body vibration on BMD and muscle strength

In **paper IV**, WBV did not improve lower extremity muscle strength, as measured by Leonardo Mechanography. The only parameter that showed improvement was the balance test in the intervention group. There are few studies to compare the current results with, which indirectly makes **paper IV** one of the first studies to be conducted on this group of patients. Regarding balance, the study by Stolzenberg [197] could not demonstrate any improvement in balance (in the single-leg stance test with eyes open) after nine months of WBV in postmenopausal women. In another study of postmenopausal women performing six months of WBV (for a mean of 8.3 min WBV per week) Russo et al. [198] showed an improvement in muscle power and velocity (measured with the single two-leg jump) compared to a control group.

There was no improvement in BMD or BMC in the calcaneus (mostly cancellous bone), which would have probably been the first location to respond to WBV stimulation as the calcaneus is the bone closest to the vibration plate. Nonetheless, other studies have shown a positive effect of WBV on bone mass measurements [119, 121, 131]. Due to the low adherence among the subjects performing the WBV training, the possibility to observe significant positive results was limited. The training setting of the study can be discussed, as the WBV training programme could have been more intense and frequent, which could have resulted in greater effects, although the variation in adherence was considerable. The intention of the in-house WBV study design was to hopefully make it easier for the subjects to perform the training, as they had the WBV plate in their own homes. Unfortunately, this subject group is very difficult to motivate and with a too extensive WBV training schedule there would be a great risk of exhausting the subjects already during the first weeks.

In **paper IV** we confirmed the hypothesis that sclerostin could be an early marker for the muscle stimulus of bone through WBV training, after which levels of sclerostin decrease and this effect has been suggested to improve osteogenesis [199]. In a study by Harrison et al. [26] sclerostin levels were

not affected after WBV in healthy pre-pubertal boys during a study period of maximum five days.

5.1 Strengths and limitations of the thesis

Strengths

In **paper I** we performed a study in very young subjects, with a mean age of 4.5 years. We assessed several relevant biochemical markers for glucose and bone metabolism in this young age group. In addition, we measured bone mass and corrected for bone size by using heel DXL and measuring BMAD.

A strength throughout this thesis is that we have evaluated all three forms of OC, namely, total OC, ucOC and cOC, in subjects with an age spectrum ranging from a mean of 4.5 years (**paper I**) to a mean of 20.4 years (**paper III**). In these same groups we have evaluated BMD and bone metabolism parameters. Furthermore, the same method and laboratory for analysing the OC forms was used in **paper II** and **paper IV**. In **paper III**, the diet intervention was extensively controlled as the subjects were undergoing inpatient treatment and observed while eating. Additionally, we used both DXA and pQCT, which enhances the reliability of the BMD measurements. The prospective randomized controlled study design in **paper IV** makes the WBV results more trustworthy. The study design in **paper IV**, in which children with obesity performed WBV, has to our knowledge not been performed earlier, except for a similar study by Erceg et al. [131].

Limitations

There are some limitations in the studies. In **paper I**, **paper II** and partly in **paper IV** the sample sizes are small, which makes it challenging to detect the hypothesized differences and correlations that otherwise could have been observed, and it also increases the probability of type I errors in the analysis. Furthermore both in **paper I** and **paper II**, the two groups were somewhat unevenly distributed regarding the number of subjects included. It would have been better if the subjects in the overweight/obese group had been more obese, and the cross-sectional study design was a limiting factor. The blood samples in **paper I** were not assessed at the same time as the heel DXL measurements because the heel DXL measurement was not part of the IDEFICS study protocol, which makes the conclusions less firm. Different laboratories and methods were used to asses OC in **paper I**, **paper II**, **paper III** and **paper IV**. An important fact when analysing the data in **paper I** was the limited possibility to analyse the three different OC methods as the

available methods were very unsecure. In **paper I**, **paper II** and **paper IV** the HOMA index was used as a parameter for insulin resistance, which is not the gold standard. Due to lack of serum, we could not measure other BTMs, such as PINP, in order to isolate the effect of OC in **paper II**. In **paper III** we did not measure a non-weight-loading body part, for example the radius, because it would have caused increased radiation at a fertile age. A control group was not included because it would be difficult to justify recruiting normal-weight young women to a restricted diet in a hospitalized environment for 12 weeks with the objective of gaining fat mass and approximately 10 kg in weight. An important limitation in **paper IV** is the lack of supervised WBV training staff, which led to a low WBV adherence rate of 51% and the subjects in the WBV group participating for only 13 minutes per week compared to the expected 21 minutes per week. Vitamin K was not assessed in any of the four studies, which is a limitation considering the fact that increasing vitamin K levels yield decreased ucOC concentrations [200].

6 CONCLUSIONS

- Throughout all four papers we could not demonstrate any clear observation that total OC, ucOC or cOC might have a positive correlation with anthropometric or biochemical parameters of glucose metabolism. The only observation that could indicate a hypothesized connection between the OC forms and anthropometric parameters was in paper II, where cOC was lower in obese subjects than in normal-weight subjects.

- In paper I, it was demonstrated that adiponectin may have an independent role in bone development in young children. However, we could not confirm any correlation between adiponectin and BMD in underweight subjects (paper III) or in subjects with obesity (paper IV).

- All three forms of OC increased during rapid weight gain in AN patients, although none of them showed any correlation with BMI or insulin as a proxy marker of glucose metabolism. In contrast to paper I, adiponectin did not show any correlation with bone measurements or BMI.

- In paper III, total BMC increased while total BMD was unchanged, as measured with DXA, which could depend on the large increase in fat mass and its interference with the DXA technique.

- Twelve weeks of home-based WBV did not affect metabolic parameters, anthropometric measurements, lower extremity muscle strength or calcaneal bone mass in paper IV. The limited sample size and the fact that the adherence rate to WBV was 51% both make it difficult to generalize. Balance improved both objectively and subjectively.

- The WBV therapy decreased the levels of sclerostin, which implies that in children with obesity, WBV has direct effects on osteocytes and indirectly on the process of bone mechanotransduction.

7 FUTURE PERSPECTIVES

To further explore the possible role of total OC, ucOC and cOC in glucose metabolism and their potential beneficial effects, independent research groups need to repeat the studies on rodents conducted by Karsenty and colleagues. These studies would either replicate the existing results or reject the suggested positive effects of OC on glucose metabolism and adiposity.

A series of studies are needed, using a longitudinal randomized controlled design with a high power to detect differences in total OC, ucOC and cOC levels between normal-weight children and children with obesity, as well as any associations with proxy markers of glucose metabolism. It is important to develop and standardize the assay kits and technical methods for detecting total OC, cOC and ucOC. When these methods are improved, comparisons between studies will be increasingly reliable.

An exciting study to perform would be to follow a large group of children longitudinally from early childhood to late adolescence and study the development of obesity in parallel to bone development and biochemical markers.

In the current thesis, the AN subjects were studied during a short period of time; it would have been interesting to follow the same group of subjects longitudinally and investigate the development of osteoporosis and fracture prevalence as well as the magnitude and stability of their weight gain. In such a study, blood samples should preferably be collected once a week, which would make it possible to closely monitor the BTM dynamics. To measure bone quality even more precisely, methods such as high resolution pQCT and microindentation should be used. In such a study it would be interesting to examine the local impact of a locally increasing amount of adipose tissue on bone quality [201]. Likewise, it would be of interest to examine local bone quality in patients with obesity who are losing weight.

In today's high-tech society, most parts of the world have access to a very stable Internet connection, and the software for video calling is quite reliable (for example Skype and Google Hangouts). Additionally, fitness centres and the personal trainer industry are growing fast in the western world. The demand for direct feedback is more widespread. This leads to the prospect of telemedicine playing a larger role in patient treatment in the future, and we already have apps that enable you to consult a physician directly via your smartphone. WBV training could be monitored from the hospital, where a

physiotherapist observes a group of four or five children while they are training in their home or running in the forest and have their smartphone or tablet with them. The physiotherapist acts as an "online personal trainer" via a group video call. This would probably increase the interest of the participants to exercise and thus increase their compliance with the exercise regime. At the other end of the weight spectrum, the development of "wearables" (wearable technology) [202] could be used in monitoring AN patients' physiological function via biofeedback; this would allow a more custom-made, individual approach during both inpatient and outpatient treatment.

In the future, priority must be given to laws and regulations to improve school nutrition, labelling of menus, adjusting the market prices for important groceries, and to some extent putting extra taxes on specific unhealthy foods [203]. These regulations have been introduced in several countries [204, 205] and will most probably be increasingly adopted into government health policies around the world.

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