Bone health and nutrition treatment

Studies on young women with anorexia nervosa and children with epilepsy

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To Edvin, Ebba, and Klara ♥

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ABSTRACT

Background: Adequate nutritional status is crucial for optimal longitudinal growth and accrual of bone mass during childhood and adolescence. Peak bone mass achieved in young adulthood affects bone health throughout life. Anorexia nervosa (AN) is associated with impaired bone health, and the cornerstone for treatment is weight restoration through nutrition treatment. For children with refractory epilepsy, the modified Atkins diet (MAD) is a treatment option to reduce the number of seizures.

The aim of this thesis was to clarify the effects of these two different nutrition treatments on bone health, body composition, and growth.

Methods and results: Two cohorts were studied: young women with AN (papers I–III) and children with therapy-resistant epilepsy (paper IV). In paper I, levels of 25-hydroxy vitamin D (25(OH)D) were evaluated in 25 young women with severe AN during 12 weeks of intensive nutrition treatment; the level was found to be adequate but decreased during treatment despite a high intake. No association was found between 25(OH)D and BMI or bone mass measured with dual-energy X-ray absorptiometry (DXA). In paper II, the role of the fat mass and obesity associated (*FTO*) gene for weight gain and body composition was evaluated, and no difference between the genotype groups (AA, TA, and TT) was observed. Paper III is a 3-year follow-up of women with AN, where bone health was assessed by DXA, peripheral quantitative computed tomography, and bone biomarkers and compared to healthy young women. BMI and body composition were stable, and there were positive changes in bone biomarkers. Despite this, trabecular density decreased, and most bone mass parameters were lower in AN subjects than in the healthy women. In paper IV, growth, and bone mass measured with DXA and with DXA and laser was evaluated in 38 children with epilepsy treated with MAD for 24 months. Of these, 53% responded well to the diet (seizure reduction >50%) and there were no negative effects on growth or bone mass.

Conclusions: A high intake of vitamin D is needed to preserve sufficient vitamin D levels during in-hospital nutrition treatment for AN. Individualized nutrition treatment for AN based on the *FTO* genotype could not be supported according to our study. AN in adolescence negatively affects bone acquisition, which is why early evaluation of bone health and a structured long-term follow-up is essential in women with AN, even in individuals who have recovered. MAD is effective for seizure reduction and can be considered a safe epilepsy treatment option in children and adolescents in terms of growth and bone health.

Keywords: intensive nutrition treatment, modified Atkins diet, vitamin D, *FTO*, bone mass, bone biomarkers, growth, skeleton, anorexia nervosa, eating disorder

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SAMMANFATTNING PÅ SVENSKA

Bakgrund och syfte

Skelettet är ett metabolt aktivt organ som genomgår kontinuerlig omsättning under hela livet. Benmassan byggs upp under barn- och ungdomsår och den maximala benmassan uppnås mellan 20 och 30 års ålder. Benbildningen regleras av olika faktorer såsom genetik, nutrition, vitamin D, hormoner och fysisk aktivitet. En minskad mineralisering av skelettet under uppväxten ger lägre maximal benmassa och ökar risken för utveckling av osteoporos och frakturer senare i livet. Tillväxten hos barn och ungdomar reflekterar allmän hälsa och nutritionsstatus och bristande nutrition kan leda till såväl tillväxtavplaning och försenad pubertet som lägre maximal benmassa.

Anorexia nervosa (AN) är en psykiatrisk sjukdom som har ett flertal allvarliga somatiska komplikationer. Kronisk undernäring tillsammans med de hormonella förändringar som den ger upphov till, kan ge minskad benmineralisering och öka risken för utveckling av osteoporos senare i livet. Behandling av AN består av en kombination av nutritionsbehandling och psykoterapi. Vid tidpunkten för dessa studier användes vid barn- och ungdomspsykiatriska kliniken, Drottning Silvias barnsjukhus, Sahlgrenska Universitetssjukhuset, en behandlingsmodell där patienter med svår AN vårdades inneliggande med ett högt energiintag redan från behandlingsstart, vilken inte hade utvärderats. Förhoppningen var att behandlingen skulle ge ett tillfrisknande från AN och leda till förbättrad benhälsa.

FTO-genen (fat mass and obesity associated gene) och dess koppling till övervikt är välkänd, men få studier har undersökt genens betydelse för ätstörningar. Syftet med en av studierna i avhandlingen var att undersöka sambandet mellan de olika genotyperna i denna gen och ökningen av vikt och fettmassa under nutritionsbehandling hos kvinnor med AN.

För barn och ungdomar med epilepsi som inte svarar på antiepileptiska läkemedel är ett behandlingsalternativ modifierad Atkins diet (MAD). Denna behandling innebär att mängden kolhydrater i kosten begränsas till ett minimum, vilket leder till fettnedbrytning och bildning av ketonkroppar, som fungerar som alternativ energikälla och som ger en minskning av anfallsfrekvensen. Tidigare studier har visat att den klassiska ketogena kosten, som är mer restriktiv, kan ge försämrad tillväxt och benmassa, men långtidsuppföljningar av barn och ungdomar med MAD-behandling saknades.

Metod

Två patientgrupper studerades: unga kvinnor med AN (delarbete I-III) och barn med terapiresistent epilepsi eller neurometabola sjukdomar (delarbete IV).

I delarbete I analyserades vitamin D och bentäthteten mättes med dual-energy X-ray absorptiometry (DXA) före och efter 12 veckors intensiv nutritionsbehandling hos 25 kvinnor med svår AN. I delarbete II analyserades *FTO*-genens betydelse för förändringen av vikt och kroppssammansättning. Delarbete III är en uppföljning utav 20 av kvinnorna med AN där bentätheten utvärderades med DXA och peripheral computed tomography och markörer för benomsättning i blodet före och efter nutritionsbehandling samt efter 3 år. I delarbete IV inkluderades 38 barn då de startade behandling med MAD. Längd och vikt följdes under 24 månader liksom blodprover (bland annat ketoner, vitamin D och tillväxtfaktorn IGF-I). Bentätheten mättes vid start, efter 12 och 24 månader med DXA och häl-DXL (DXA och laser).

Resultat

Förekomsten av D-vitaminbrist var låg, men D-vitaminnivåerna sjönk under behandlingsperioden trots att det totala intaget av D-vitamin genom mat och kosttillskott var högt. Det fanns inget samband mellan D-vitaminstatus och BMI eller bentäthet hos kvinnorna med AN. BMI ökade från 15,4 till 19,0 kg/m^2 och total fettmassa från 14% till 27% av kroppsvikten under nutritionsbehandling, det fanns dock ingen skillnad i förändring av BMI eller kroppssammansättning mellan de olika genotypgrupperna av *FTO*-genen (AA, TA eller TT). Vid uppföljning efter 3 år var BMI och kroppssammansättning stabila och markörerna för benomsättning förändrades på ett för skelettet fördelaktigt sätt (CTX och sklerostin minskade samtidigt som BALP ökade). Trots detta ökade inte bentätheten mätt med DXA hos kvinnorna med AN och densiteten hos det trabekulära benet (mätt med pQCT) minskade. De flesta bentäthetsparametrar var lägre hos kvinnorna med AN än hos en grupp friska unga kvinnor efter 3 år. Behandling med MAD hade god effekt (>50% minskning av anfallsfrekvensen) hos 53% av individerna. BMI förbättrades, kroppssammansättningen var stabil och längdtillväxten påverkades inte av behandling med MAD under 24 månader. Bentäthetparametrarna var stabila under behandlingsperioden.

Slutsatser

Till följd av ökad fettmassa och minskad solexponering minskade D-vitaminnivåerna hos kvinnor med AN under behandlingsperioden, trots adekvat intag av D-vitamin. Därmed kan ett högt intag av D-vitamin under nutritionsbehandling för AN rekommenderas, för att hålla normala nivåer och

optimera förutsättningarna för benmineralisering. Individanpassad nutritionsbehandling utifrån *FTO*-genen kan inte stödjas utifrån dessa resultat. Fler och större studier behövs för att utforska sambandet mellan *FTO*-genen och ätstörningar. AN under de viktiga åren i ungdomen, då en stor del av benmassan byggs upp, ger en minskad benmineralisering och lägre maximal benmassa jämfört med friska individer, även hos de individer som tillfrisknat från AN. Mätning av bentätheten bör utföras så snart ätstörning misstänks hos en ung kvinna och bör sedan följas upp regelbundet under lång tid, för att långsiktiga konsekvenser som osteoporos och frakturer ska kunna förbyggas.

Tillväxten och benmineraliseringen påverkades inte av behandling med MAD, vilket sannolikt beror på ökat BMI och stabil kroppsammansättning, tillskott av vitaminer och mineraler samt lägre ketosnivå jämfört med behandling med den klassiska ketogena kosten. MAD kan anses vara en säker behandling för barn och ungdomar vad gäller tillväxt och bentäthet, utifrån studien i denna avhandling.

LIST OF PAPERS

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

I. **Svedlund A**, Pettersson C, Tubic B, Magnusson P, Swolin-Eide D. Vitamin D status in young Swedish women with anorexia nervosa during intensive weight gain therapy. *European Journal of Nutrition 2017; 56: 2061–2067*.

II. **Svedlund A**, Tubic B, Elfvin A, Magnusson P, Swolin-Eide D. The significance of the *FTO* gene for weight and body composition in Swedish women with severe anorexia nervosa during intensive nutrition therapy. *Journal of the American College of Nutrition 2022; 41: 594–599,*
DOI: 0.1080/07315724.2021.1945979 (New journal name from 2022: Journal of the American Nutrition Association).

III. **Svedlund A**, Pettersson C, Tubic B, Ellegård L, Elfvin A, Magnusson P, Swolin-Eide D. Bone mass and biomarkers in young women with anorexia nervosa: a prospective 3-year follow-up study. *Journal of Bone and Mineral Metabolism 2022; 40: 974–989*.

IV. **Svedlund A**, Hallböök T, Magnusson P, Dahlgren J, Swolin-Eide D. Prospective study of growth and bone mass in Swedish children treated with the modified Atkins diet. *European Journal of Paediatric Neurology 2019; 23: 629–638*.

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ADDITIONAL RELEVANT PAPERS NOT INCLUDED IN THIS THESIS

I. Pettersson C, Tubic B, **Svedlund A**, Magnusson P, Ellegård L, Swolin-Eide D, Forslund HB. Description of an intensive nutrition therapy in hospitalized adolescents with anorexia nervosa. *Eating Behaviors 2016; 21: 172–178*.

II. Tubic B, Pettersson C, **Svedlund A**, Bertéus Forslund H, Magnusson P, Swolin-Eide D. Increased bone mineral content during rapid weight gain therapy in anorexia nervosa. *Hormone and Metabolic Research 2016; 48: 664–672*.

III. Novak D. Forsander G, Kristiansen K, **Svedlund A**, Magnusson P, Swolin-Eide D. Altered cortical bone strength and lean mass in young women with long-duration (19 years) type 1 diabetes. *Scientific Reports 2020; 10: 22367*.

IV. Pettersson C, **Svedlund A**, Wallengren O, Swolin-Eide D, Paulson Karlsson G, Ellegård L. Dietary intake and nutritional status in adolescents and young adults with anorexia nervosa: a 3-year follow-up study. *Clinical Nutrition 2021; 40: 5391–5398*.

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ABBREVIATIONS

$1,25(OH)D_2$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
aBMD	Areal bone mineral density
ALP	Alkaline phosphatase
AN	Anorexia nervosa
ANCOVA	Analysis of covariance
BALP	Bone-specific alkaline phosphatase
BMAD	Bone mineral apparent density
BMC	Bone mineral content
BMI	Body mass index
CI	Confidence interval
CLIA	Chemiluminescence immunoassay
CTX	Carboxy-terminal crosslinked telopeptides of type I collagen
CV	Coefficient of variation
DBP	Vitamin D binding protein
DSM	Diagnostic and Statistical Manual of Mental Disorders
DXA	Dual-energy X-ray absorptiometry
DXL	Dual-energy X-ray absorptiometry and laser
FTO	Fat mass and obesity-associated gene
GH	Growth hormone

GLUT1-DS	Glucose transporter type 1 deficiency syndrome
GWAS	Genome-wide association study
IGF-I	Insulin-like growth factor I
IGF-BP3	Insulin-like growth factor binding protein 3
IPAQ	International Physical Activity Questionnaire
ISCD	International Society for Clinical Densitometry
KD	Ketogenic diet
MAD	Modified Atkins diet
NHANES	National Health and Nutrition Examination Survey
n-oxPTH	Non-oxidized parathyroid hormone
PBM	Peak bone mass
PCR	Polymerase chain reaction
PDHCD	Pyruvate dehydrogenase complex deficiency
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone
SD	Standard deviation
SDS	Standard deviation score
SNP	Single nucleotide polymorphism
TBLH	Total body less head
vBMD	Volumetric bone mineral density

DEFINITIONS IN SHORT

Low bone mass	Areal bone mineral density (aBMD) Z-score ≤-2 SD
Osteoporosis in children	Presence of a vertebral compression fracture (in the absence of high-energy trauma or local disease), or a significant fracture history comprising ≥2 long bone fractures by age 10 years or ≥3 long bone fractures by age 19 years, combined with low aBMD Z-scores (≤-2 SD) [1]
Drug-resistant epilepsy	Failure to achieve sustained seizure freedom with two anti-epileptic drugs [2]

THESIS AT A GLANCE

Paper	**I**	**II**	**III**	**IV**
Aims	To assess vitamin D status and its association with BMI, fat mass, and bone mass in AN	To study the relations of the *FTO* gene with body composition and BMI in AN	To investigate bone mass, bone turnover markers, and body composition long-term in AN	To assess bone mass, growth, and body composition in children on MAD
Methods				
Study design	Prospective cohort study	Prospective cohort study	Prospective cohort study	Prospective cohort study
Population and inclusion criteria	Women with AN (*n*=25), age 16–24 years, admitted for intensive nutrition treatment for 12 weeks	Women with AN (*n*=25), age 16–24 years, admitted for intensive nutrition treatment for 12 weeks	Three-year follow-up of individuals from papers I and II (*n*=20), matched with a healthy comparison group (*n*=17)	Children with intractable epilepsy (*n*=38) aged 0.4–17.2 years, admitted for treatment with MAD
Study period	2012–2014	2012–2014	2012–2017	2010–2017
Results	Vitamin D status was adequate, but decreased, during treatment and was not correlated to BMI or bone mass parameters	No difference in weight, BMI, or fat mass between the *FTO* genotype groups	Compromised bone mass in AN, despite stable BMI and body composition, and positive changes in bone turnover markers	No negative effect on longitudinal growth or bone mass during treatment with MAD
Conclusions and clinical significance	A high intake of vitamin D is needed during intensive nutrition treatment in hospitalized AN patients	Individualized nutrition treatment for AN based on the *FTO* genotype is not supported	Early evaluation and structured follow-up of bone health is warranted, even in women who have recovered from AN	MAD is a safe treatment regarding bone health and growth in children and adolescents with epilepsy

1 INTRODUCTION

Adequate nutritional status is crucial to maintain optimal longitudinal growth and accrual of bone mineral during childhood and adolescence. The maximum peak bone mass (PBM) achieved in young adulthood is of great importance and affects long-term bone health throughout life. PBM is to a large extent determined genetically but is also affected by nutrition, hormonal factors, physical activity, and exposure to chronic diseases or medications. Some of these factors are modifiable and hence it is possible to optimize the conditions for the growing skeleton to achieve its full genetic potential.

Anorexia nervosa (AN) is a psychiatric illness with several somatic complications related to chronic starvation and the accompanying endocrine dysregulation. One long-term consequence of AN during adolescence is impaired bone acquisition and thus increased risk for osteoporosis and fractures later in life. Treatment of AN aims at weight recovery and normalized eating behavior through a combination of nutrition treatment and psychotherapy. At the Child and Adolescent Psychiatry department at the Queen Silvia Children's Hospital, Gothenburg, Sweden, patients with severe AN were treated as inpatients for 12 weeks with a nutrition treatment comprising high energy from the start, a treatment model that had not yet been evaluated. The objective of the studies was to evaluate different aspects of bone health and body composition in young women with AN during this specific nutrition treatment and at follow-up after 3 years.

For children with intractable epilepsy or neurometabolic conditions, one treatment option is the modified Atkins diet (MAD), which aims to reduce seizures and improve the quality of life. There was a concern that this nutrition treatment would involve side effects affecting growth and bone mass in these patients, who are often already exposed to other factors impairing bone health such as limited mobility. MAD treatment was a part of clinical practice at the Department of Neurology, Queen Silvia Children's Hospital, but long-term studies on children receiving MAD treatment were lacking. Our intention was thus to generate new insights into longitudinal growth and bone mass in children on this nutrition treatment.

2 BACKGROUND

2.1 GROWTH

Human longitudinal growth is regulated by several factors: genotype, nutrition hormones, and growth factors. Linear growth can be illustrated by the ICP growth model, where growth during childhood and adolescence is divided into three phases – infancy (I), childhood (C), and puberty (P) (figure 1) [3]. These periods are characterized by distinct features, and the regulatory mechanisms differ between them.

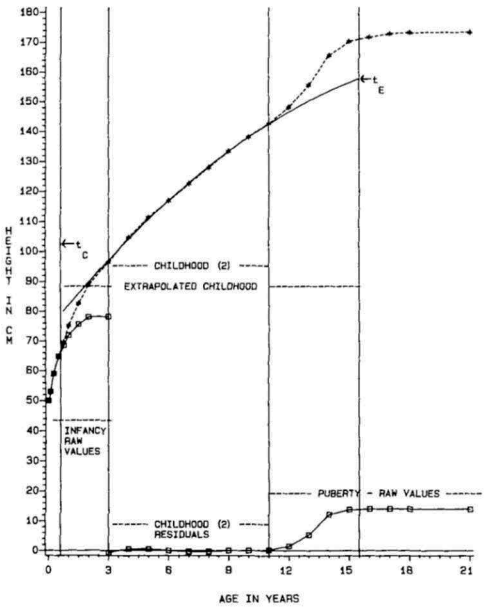


Figure 1. The ICP model for boys. Reproduced from Karlberg. On the construction of the infancy-childhood-puberty growth standard. Acta Paediatr Scand Suppl 1989: 356; 26-37. Reprinted with permission from John Wiley and Sons.

During the infancy phase, nutrition is the principal regulator of growth together with thyroid hormone, with a minimal input from growth hormone (GH). From approximately 6 months of age the childhood phase starts. During this phase, which predominates from 3 years of age, hormonal influences become the major regulators, especially the GH-insulin-like growth factor axis and thyroid

hormones, and nutrition becomes less important. Accordingly, dysfunction in the GH axis may be recognized during this period. During puberty, the growth pattern changes dramatically and differs between girls and boys. The development of primary and secondary sex characteristics during puberty is defined by the Tanner scale, with stages from I (prepubertal) to V (fully developed) [4]. Peak height velocity in girls occurs at approximately 12 years of age and coincides with breast development, the first sign of puberty. In boys, the growth spurt starts approximately two years later and coincides with testicular volume 10–12 ml, when puberty is well established (Tanner stage III).

2.1.1 NUTRITION

Evaluation of the nutritional intake of a child is important, in order to establish whether it is adequate to meet the child's current needs related to growth pattern, developmental level, and the clinical situation [5]. Since energy required for growth constitutes a small percentage of total energy expenditure, this means that the food or energy intake must be severely restricted to cause growth failure [6]. Psychosocial deprivation is another potent cause of growth failure that should always be considered in children and adolescents. For these reasons, growth monitoring is an essential part of child health surveillance programs and growth should be evaluated using age- and gender-adjusted growth charts [7]. Height between -2 SDS and +2 SDS on the growth chart is considered normal; however, a growth pattern decelerating or deviating markedly from mid-parental height is considered pathological.

Reference values for energy requirements in children and adolescents are based on resting energy expenditure, physical activity level, and energy requirements for growth. Values for resting energy expenditure, based on either body weight or a combination of weight and height, are first calculated and then multiplied by an appropriate physical activity level (low, average, or high) [8]. During starvation, the resting energy expenditure decreases as a result of weight loss and decreased muscle mass, and to the metabolic adaptation related to, for instance, alterations in thyroid hormones [9]. In adults, the reference values for energy requirements are based on resting energy expenditure and physical activity level. Low energy intake in adults is defined as an energy intake of 1550–1900 kcal/day and very low energy intake as an energy intake below 1550 kcal/day, with increased risk of insufficient intake of micronutrients [8]. Tables 1 and 2 describe estimated energy requirements and recommended nutrient intake in children and adults, according to the Nordic Nutrition Recommendations [8], which constitute the basis for the composition of the nutrition treatments described in this thesis.

Table 1. Estimated daily energy requirements based on resting energy expenditure and an average physical activity level [8].

Age (years)	Gender	Mean Body weight (kg)	Energy requirements (kcal)
2	Girls Boys	12.5 13.2	990 1070
5	Girls Boys	19.2 19.4	1400 1490
10	Girls Boys	31.8 32.2	1980 2140
15	Girls Boys	53.2 55.1	2340 2760
18–30	Women Men	64.4 75.4	2250 2800

Table 2. Recommended dietary composition of nutrients and recommended daily intake of vitamin D and calcium [8].

	Children 2–17 years	Adults 18–64 years
Carbohydrates, E%	45–60	45–60
Protein, E%	10–20	10–20
Fat, E%	25–40	25–40
Vitamin D, μg/day	10	10
Calcium, mg/day	600 (2–5 years) 700 (6–9 years) 900 (10–17 years)	800

(E% = percent of total energy intake)

2.1.2 THE GH-IGF-I AXIS

The major endocrine regulator of linear growth is GH, which is secreted by somatotroph cells of the anterior pituitary gland. The hormone is secreted in pulses, regulated by the hypothalamic peptides somatostatin and GH-releasing hormone, and it circulates in blood bound to GH binding protein. Many other factors influence GH secretion, including genetic factors, estrogen and testosterone, thyroxine, glucocorticoids, fasting, physical exercise, stress, and sleep [6].

Insulin-like growth factor-I (IGF-I) is a peptide hormone that is produced predominantly by the liver in response to GH and is involved in a wide variety of physiological processes; among these, IGF-I is a crucial factor for bone formation and longitudinal growth through stimulation of proliferation and differentiation of chondrocytes in the epiphyseal plates [10, 11]. The concentration of circulating IGF-I is determined by and closely related to the physiological secretion of GH. IGF-I binds to IGF binding proteins, especially IGFBP-3, the production of which is also strongly GH dependent. IGF-I exerts endocrine effects on target tissues, including bone, and is also synthesized by osteoblasts, exerting a paracrine effect on adjacent cells [12].

IGF-I is regulated by nutrition, with hepatic production facilitated by dietary proteins [10], and accordingly, the serum concentration of IGF-I falls with malnutrition, with the purpose of diverting substrates towards the body's energy needs. Secondary to this, there is reduced negative feedback on GH secretion, resulting in increased GH levels. Malnutrition is also related to a state of relative GH insensitivity, which is a part of the adaptive response, resulting in persistently low IGF-I concentrations [13]. During puberty in both girls and boys, estradiol is an important hormone influencing the GH-IGF axis. Small-to-moderate levels of estradiol stimulate the hypothalamus to increase GH-releasing hormone secretion, resulting in increased GH secretion. This, in turn, leads to increased IGF-I levels, accelerating growth. Higher estrogen levels, however, inhibit growth through action on the growth plate, which therefore causes the termination of linear growth in both sexes.

2.2 BODY COMPOSITION

Body composition describes the proportions of fat mass and fat-free mass (lean mass), the latter including all soft tissue except fat mass. It is important to monitor body composition in order to evaluate the effects of diet, illnesses, growth, and physical activity on body stores. Different techniques can be used for the analysis of body composition, from basic anthropometric measurements to advanced imaging techniques. One of the simplest and most widely used methods to evaluate the nutritional status is BMI (weight/height^2). However, this measure, does not distinguish fat mass from lean mass. Also, BMI SDS has low specificity in identifying changes in fat mass percentage [14]. Since the proportion of fat mass and lean mass varies by age, gender, and hormonal maturation during childhood, this is particularly relevant in the pediatric population [15]. With dual-energy X-ray absorptiometry (DXA), measures of fat mass and lean mass are provided. The recommendation is to use fat mass percentage or fat mass and lean

mass index (kg/m^2) for the evaluation of nutritional status in children and adolescents [16, 17]. Another option for measuring body composition that has gained popularity is bioelectrical impedance analysis, a simplified technique that calculates fat-free mass using a chemical model and mathematical equations; fat mass is then calculated by subtracting fat-free mass from total body weight. However, the validity of this method in children and adolescents needs to be improved [18].

Lifestyle factors and body composition influence bone development in childhood and eventually co-determine the PBM attained during early adulthood [19, 20]. The majority of fat mass and lean mass are acquired during adolescence [21]. In a review by Sioen et al. [22] it is clarified that the contribution of lean mass to the variance of bone mass parameters is larger than the contribution of fat mass. Further, it is stated that the correlation between lean mass and bone mass parameters is generally positive, which can be explained by the adaptation of bone to the peak forces caused by muscles. Hence, the increase in bone mass seen in overweight and obese children and adolescents is related to an increase in lean mass rather than fat mass.

The adipokine leptin has an important role in mediating energy expenditure, caloric intake and thus regulation of body weight and body composition. Leptin is a regulator of kisspeptin neurons in the arcuate nucleus of hypothalamus. Kisspeptin signaling is thought to be an important component of the pulsatile GnRH secretion [23]. Since leptin is mainly secreted by white adipose tissue, levels are highly correlated with body fat mass in both children and adults [24] but also reflect acute changes in caloric intake, such as fasting and overfeeding [25]. Leptin is involved in bone metabolism through both central effects and peripheral pathways involving interaction with bone marrow mesenchymal stem cells, chondrocytes, osteoblasts, and osteoclasts [26].

2.2.1 THE *FTO* GENE

The variation in BMI in the general population is largely related to genetic factors, and nearly half of the heterogeneity can be explained by heritability [27]. Through genome-wide association studies (GWAS), more than 1100 independent loci related to obesity have today been identified [28]. The fat mass and obesity associated gene (*FTO*), located on chromosome 16, was the first obesity susceptibility gene identified, in 2007 [29, 30]. Two GWAS identified different single nucleotide polymorphisms (SNPs) in the first intron of the *FTO* gene associated with BMI (rs9939609 [29] and rs9930506 [30]). *FTO* is mainly

expressed in the hypothalamus and is of great importance for the regulation of energy homeostasis. The association of *FTO* with BMI is related to changes in fat mass, and individuals homozygous for the A allele at rs9939609 are at substantial risk of being overweight (OR 1.38) or obese (OR 1.67) compared to those who are homozygous for the T allele [29].

Multiple neighboring SNPs also show significant associations with BMI. In addition to *FTO*, over 75 obesity susceptibility loci have been identified, but the *FTO* gene is most common in the European-ancestry population and has the greatest importance for obesity. Each additional risk allele of the *FTO* locus is associated with 0.39 kg/m^2 higher BMI and a 1.2-fold increased risk of obesity [31]. The association between *FTO* and obesity has also been demonstrated in children, with some effect on weight in early childhood and the largest effect in young adulthood [31]. The mechanisms behind the association between *FTO* and obesity have gained great interest. Different studies have found associations between the risk allele of *FTO* and increased intake of energy, fat, or protein [32, 33], reduced satiety and increased appetite [34], loss of control over eating [35], and poor eating habits and food choices [30]. Several studies have found that physical activity can attenuate the effect of *FTO* on BMI [36, 37], but a study focusing on the relation between *FTO* genotype and energy expenditure could not verify such relationship [32]. Thus, increasing evidence suggests that food intake might mediate the association between *FTO* and obesity, and that physical activity attenuates this association.

In the past three decades, knowledge about the genetic architecture of AN has increased. Genetic correlations have also been observed between AN and obesity, suggesting that some genetic factors influencing the variation in BMI are shared [38]. Previous studies have found that the *FTO* gene is upregulated during food deprivation and that its expression is correlated with appetite-stimulating peptides [39]. Data on the role of the *FTO* gene for AN is, however, scarce and results are incongruous. Some studies have found an association between *FTO* variants and AN, bulimia nervosa, or binge eating [40, 41], while others have not [42, 43].

2.3 BONE TISSUE

Bone is a highly dynamic organ, with a complex structure, that undergoes constant remodeling. Bone has several mechanical functions, such as providing shape and rigidity, protection and support for body structures, and aiding locomotion. Mineralization makes bone hard and resistant, distinguishing bone from other connective tissue. Bone is also a metabolically active tissue and serves as a

repository of calcium, phosphate, and magnesium, and it is intricately involved in the homeostasis of these minerals. It also serves as a reservoir for lipids and bone marrow. The mechanical strength of bone is determined by several structural elements, the most important being the bone size and geometry (cortical thickness, porosity, trabecular bone morphology), the micro and macro architecture, mineralization, and construction of the matrix [44]. The bone matrix consists of a tight network of type I collagen fibers, in which there are regions where calcium hydroxyapatite crystals will form. Bone mineralization requires the presence of sufficient concentrations of calcium, phosphate, and vitamin D. Deficiency in any of these elements can therefore cause softening of the bones, or osteomalacia, which in children is known as rickets [45]. Involvement of enzymes such as alkaline phosphatase (ALP) is also required for mineralization process.

There are two main histological types of mature bone: cortical and trabecular. Cortical bone is compact and found in the shaft of long bones and on the surface of flat bones, whereas trabecular bone is more porous and located in the metaphyses of long bones, within irregular bones such as the spinal vertebrae and calcaneus, and the inner parts of flat bones [46]. The total bone mass consists to a large extent of cortical bone (approximately 80%). However, trabecular bone comprises a large percentage of the vertebrae (66% of lumbar vertebrae and 75% of thoracic vertebrae). In the femoral neck and distal radius, the percentage of trabecular bone is 25% and 5%, respectively. The cortical bone has an outer membrane called periosteum, which is divided into an outer and an inner layer, where new bone is formed. The inner layer of the cortex is called endosteum and is the place where bone resorption takes place (figure 2). Both layers contain osteoblasts and osteoclasts. By their bone forming and resorbing functions, these bone cells together carry out remodeling, repair, and growth of the bone.

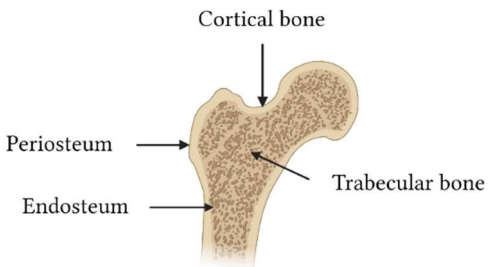


Figure 2. Human femur. Created with BioRender.com

Bone cells and fat cells originate from the same mesenchymal precursor cell within the bone marrow, which differentiate into osteoblasts or adipocytes [47]. The bone marrow adipocyte is a specific adipocyte that has the potential to interact with bone and hematopoietic cells [48]. Both aging and AN are conditions associated with increased bone marrow adipose tissue, which contributes to the decreased bone strength [48, 49], and marrow adipose tissue is inversely associated with areal bone mineral density (aBMD) in AN patients [50]. Marrow adipocytes also function in an endocrine manner by secreting adipokines, such as adiponectin [51].

2.3.1 BONE MODELING AND REMODELING

The purpose of bone modeling (construction) and remodeling (reconstruction) during growth is to establish the PBM, while the aim in adulthood is to maintain bone strength [52]. These processes aim at adjusting bone architecture based on changing mechanical needs and contribute to bone mass changes in adults, fracture healing, and repair of microdamage. During childhood, bone formation exceeds resorption and the bone turnover is high. In young adulthood, formation and resorption are balanced and bone mass is stable, but with aging, bone loss begins, caused by increased resorption and decreased formation. After the achievement of PBM, there is a stabilization of remodeling, and bone mass remains stable until the age-related bone loss begins, a process characterized by increased resorption and reduced bone formation (figure 2).

Bone density is determined by PBM and the degree of bone loss, while the quality of bone reflects the architecture, mineralization, bone turnover, and microfractures [53]. Osteoporosis is characterized by low bone mass due to low PBM, increased resorption, decreased formation (or a combination), deteriorated bone architecture, and hence increased bone fragility. The immediate consequence of osteoporosis is fragility fractures. (For a definition of osteoporosis see section 2.3.4.)

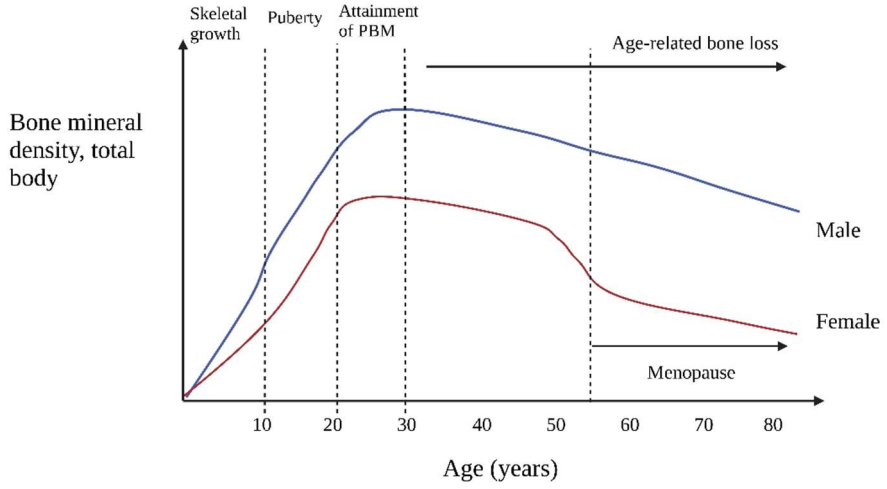


Figure 3. Bone mass throughout life. Created with BioRender.com.

Bone modeling

Modeling is the process in which new bone is deposited without previous bone resorption, which changes the size and shape of bone. Hence, bone modeling does not replace old bone. Both osteoblasts and osteoclasts are involved in this process, independently of each other. Bone modeling defines skeletal development and growth but is a process that continues throughout life. Bone modeling is regulated by genetic factors in combination with physical activity and other environmental factors, and hormonal factors (PTH and sclerostin) [54] and it is well recognized that weight bearing activities induce mechanical stress that stimulate osteoblast activity.

Bone remodeling

The predominant process in the mature adult skeleton (>30 years) is remodeling, which is a continuous and lifelong process in which mineralized bone is broken down by osteoclasts with accompanying formation of the bone matrix by osteoblasts [53, 55]. The purpose of remodeling is the replacement of old or damaged bone with new bone, thereby maintaining mechanical strength, and the maintenance of mineral homeostasis. The resorption phase lasts for 4–6 weeks [56], while the bone formation phase takes approximately 4 months [57]. The remodeling process consists of four phases (figure 4). During the *activation phase*, osteoclasts are recruited to resorb bone during the following *resorption phase*. This phase is followed by the *reversal phase*, during which the osteoclasts undergo apoptosis and osteoblasts are recruited. Lastly, during the *formation phase*, osteoblasts synthesize the new bone matrix that is subsequently mineralized [54]. Since trabecular bone has a larger area exposed to surrounding tissue and bone marrow, trabecular bone undergoes a larger bone turnover. Hence, although trabecular bone comprises a smaller proportion of total bone (20%), the bone remodeling activity predominantly takes place here [58]. Accordingly, the rate of bone turnover is highest for example in the vertebrae, and lowest where cortical bone predominates, such as the hip [59]. In the cortical bone, remodeling takes place at the endocortical and periosteal surfaces as well as inside the compact cortical bone. With aging, the trabecular bone is reduced and the importance of cortical remodeling increases.

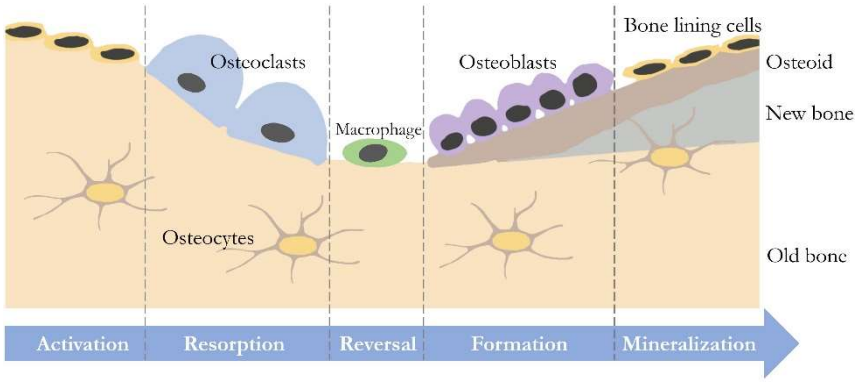


Figure 4. The bone remodeling process.

2.3.2 BONE CELLS

Osteoblasts

Osteoblasts originate from mesenchymal stem cells and represent approximately 5% of bone cells. These cells are specialized bone-forming cells that synthesize the bone matrix and regulate mineralization. Osteoblasts differentiate into osteocytes or bone lining cells and are also involved in the differentiation of osteoclasts, but whether they control osteoclastic activity is not so well understood [60]. One of the most important pathways regulating bone formation in osteoblasts is the wnt/β-catenin signaling pathway. Several inhibitors of this pathway exist, for example sclerostin. New bone is produced by osteoblasts by synthesis and secretion of type I collagen, the major bone matrix protein [61]. Phosphates released from the matrix vesicles, together with calcium from the extracellular fluid, form the hydroxyapatite crystals that enable mineralization of the bone.

Osteoclasts

Osteoclasts are multinucleated giant cells residing on the endosteal surfaces within the structural and functional units of cortical bone (the Haversian system or osteons), and beneath the periosteum on the periosteal surface. The osteon includes osteocytes, lamellae, the lacunocanalicular network, and Haversian canals, and it plays an important role in bone mechanics and turnover [62]. The primary function of the osteoclast is bone resorption, but it also participates in the regulation of osteoblastic bone formation through bone-matrix-derived factors [60]. Receptor activator of nuclear factor kappa-B ligand (RANKL), produced by osteoblasts and osteogenic stem cells, is the main stimulating factor for osteoclast development, maturation, and survival [63]. Increased RANKL expression stimulates resorption and bone mass loss.

Osteocytes

Osteoblasts terminally differentiate into osteocytes, which constitute over 90% of adult bone cells and form a cellular network throughout the hard tissue matrix of bone. The total number of osteocytes in the human skeleton (42 billion) is of the same magnitude as the number of neurons in the brain [64]. Osteocytes incorporate in the lacunae in the newly formed bone matrix and reside there for long periods, playing a central role in maintaining bone structure, before finally undergoing apoptosis [65]. Osteocytes are the primary mechanosensory cell type of bone, capable of initiating bone formation as a result of microdamage. Osteocytes located near the place of microdamage undergo apoptosis, leading to enhanced RANKL production and increased osteoclast formation, which in turn

leads to increased bone remodeling [66]. Through the secretion of sclerostin, a negative regulator of osteoblastic bone formation, osteocytes also control osteoblasts [67]. In addition, osteocytes function as endocrine cells, releasing into the circulation FGF23, a growth factor that regulates phosphate homeostasis at the kidney, and osteocalcin, affecting both cognition, glucose metabolism, and muscle [67].

2.3.3 PEAK BONE MASS

Bone deposition and resorption during growth allows the bones to expand and lengthen, a process that begins during fetal life and continues until epiphyseal fusion [68]. PBM is defined as the bone mass at the time point when longitudinal growth is completed and bone mass has reached a plateau or maximum level [69]. PBM is genetically determined (60–80%) but is also affected by hormonal factors, nutrition and adequate dietary intake of calcium, vitamin D, and protein, endocrine factors, weight-bearing activities, as well as chronic disease or medications that affect bone [19, 44]. Mechanical loads, such as physical activity or increased body weight, cause strains that activate mechanosensitive cells (osteocytes) that start a signaling process, in turn activating osteoclasts and osteoblasts [70]. Regular physical activity in adolescence and young adulthood have been related to higher PBM of the spine at the age of 27 years [71].

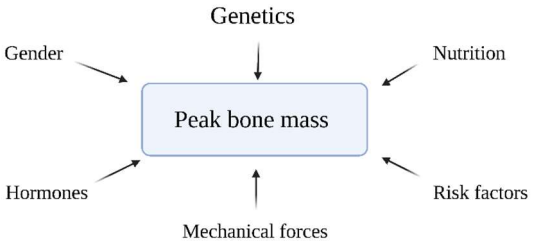


Figure 5. Factors determining peak bone mass. Created with BioRender.com.

The bone mass achieved throughout childhood and adolescence is perhaps the main determinant of skeletal health and risk of osteoporosis throughout life and it is predicted that the onset of osteoporosis could be delayed by 13 years by a 10% increase in PBM [72]. Through childhood, bone mass is acquired relatively slowly, but during the pubertal growth spurt in height, bone mineral accrual is

rapid and peaks shortly after peak height velocity: for total body, bone mineral accrual peaks at 12.5±0.90 years in girls and 14.1±0.95 years in boys [73]. The increase in trabecular bone occurs mainly between Tanner stage 3 and 4 [74], while cortical bone density increases more rapidly by the end of puberty and continues into the third decade of life [75]. The increase in bone mass slows dramatically by 16–18 years in girls and 17–20 years in boys, but the age at which PBM is achieved is a subject of debate. In a review by Davies et al. [76], some studies suggested a peak for adult women between 30 and 35 years and others suggested a peak between 25 and 35 years. A longitudinal study estimated the plateau for PBM of total body to be reached approximately 7 years after peak height velocity in both girls and boys, which equates to chronological age 18.8 years for women and 20.5 years for men. For lumbar spine the plateau was reached 5 years and for hip 3 years after peak height velocity [77]. In contrast to this, the Canadian Multicenter Osteoporosis Study identified the ages for lumbar spine PBM to 33–40 years in women and 19–33 years in men; the corresponding ages for total hip PBM were 16–19 years in women and 19–21 years in men [69]. Using peripheral quantitative computed tomography (pQCT), Riggs et al. [75] demonstrated that 20–29-year-old women were losing trabecular bone at distal radius and lumbar spine (1–1.75% per year) but gaining cortical bone (0.25% per year).

Effects of disease, medications, hormonal changes, nutritional status, and physical activity during the period of rapid bone accrual can thus have major consequences for long-term bone health. The consequences of postmenopausal or age-related bone loss on fracture risk depends on PBM and the rate of bone loss, with each SD of bone lost from PMB later in life doubling the fracture risk [19]. Thus, optimizing PBM is of utmost importance for the prevention of osteoporotic fractures later in life, but also for reducing fracture incidence during puberty.

A satisfying nutritional status is essential for both growth and bone health. Calcium is the most important nutrient for achieving PBM and for preventing bone loss. Vitamin D is required for optimal calcium absorption and is accordingly important for bone health, (see section 2.3.7 on vitamin D). Vitamin D deficiency increases parathyroid hormone (PTH), leading to mobilization of calcium from bone and thereby reduced bone mass. Besides calcium and vitamin D, dietary protein is a key nutrient for bone health. Since dietary protein stimulates the production of IGF-I, low protein intake may negatively affect the acquisition of bone mass during growth [10]. In healthy individuals, a positive relationship between IGF-I and accrual of bone mineral content (BMC) during puberty has been shown [78].

Up to 50% of all children sustain a fracture between 5 and 18 years of age, the risk being higher in boys than in girls [19]. In both boys and girls, the incidence of fractures peaks around the period of peak height velocity [58], and the most common site is the distal radius/ulna in both sexes [19]. The skeleton is particularly vulnerable to fractures during this period of rapid growth, due to lower volumetric BMD (vBMD) and thinner cortical bone with increased cortical porosity [70]. Children with fractures have lower BMC and bone size than controls who have not had fractures [79]. Although weight-bearing activities are critical for bone acquisition and PBM, vigorous physical activity is a risk factor for fractures during childhood, independent of bone mass [80].

2.3.4 OSTEOPOROSIS

Osteoporosis is a medical condition characterized by bone fragility related to low bone mass and microarchitectural disruption, predisposing a person to increased fracture risk. Osteoporosis is common and caused 4.3 million fragility fractures in Europe in 2019, mostly in women [81]. Osteoporotic fragility fractures in adults are most common in bones rich in trabecular bone, such as vertebrae, hip, and wrist. Primary osteoporosis often follows menopause in women, because of reduced estrogen production, and occurs later in life in men. Secondary osteoporosis is, however, a result of diseases (e. g. endocrine, nutritional, gastrointestinal), hypogonadism, or medications (e.g. glucocorticoids) [53]. For postmenopausal women and men older than 50 years, osteoporosis is defined by the World Health Organization as an aBMD T-score (difference in SD from the mean value of young adults) of ≤-2.5 SD measured by DXA [82].

In the pediatric population, however, the DXA results are expressed as Z-scores calculated from age- and gender-adjusted reference data that is available from 5 years of age. Primary osteoporosis in children is caused by genetic defects such as in osteogenesis imperfecta, whereas secondary osteoporosis is caused by nutritional deficits, AN, immobilization and low muscle mass, hormonal deficiencies, inflammatory diseases, or medications [83]. The International Society for Clinical Densitometry (ISCD) has stated that the diagnosis of osteoporosis in children should not be based upon densitometric criteria alone [1]. If a vertebral compression fracture is present, in the absence of local disease or high-energy trauma, the diagnostic criteria for osteoporosis are met, irrespective of aBMD. However, in the absence of a vertebral compression fracture, the diagnosis of osteoporosis requires a clinically significant fracture history, namely, ≥2 long bone fractures at age 10 years, or ≥3 long bone fractures at 19 years, combined with low aBMD Z-scores (≤-2 SD) measured by DXA [84, 85]. Vertebral compression

fractures are rare in the pediatric population and often associated with an underlying bone fragility disorder. In non-ambulatory children, up to 50% of fractures are femur fractures [86]. Higher aBMD Z-scores (>-2 SD) do not exclude the possibility of skeletal fragility. With the ICSD definition, over-diagnosis of osteoporosis in healthy children who sustain fractures during play can be avoided. However, there is a risk of missing clinically relevant bone fragility in some children if fracture characteristics (skeletal site and radiographic elements) or the clinical picture, for example serious illness, are not also considered [87].

2.3.5 MARKERS OF BONE TURNOVER

Markers of bone turnover reflect the activity and number of osteoclasts (bone resorption markers) and osteoblasts (bone formation markers). Several markers can be measured with good precision but are sensitive to, for example, recent fractures. In healthy adolescents, both formation and resorption markers are at high levels related to rapid bone growth, mineralization, and remodeling, whereas the levels decrease to adult levels during late puberty [88]. Bone turnover markers are not used clinically to diagnose osteoporosis or to predict fracture risk in an individual, but in patients with osteoporosis, such markers can be used to assess the effect of antiresorptive treatment [56]. In pediatrics, however, bone turnover markers are still mainly used for research.

Bone formation markers

Bone formation markers mirror the different phases of osteogenesis. ALP is an enzyme that transforms inorganic pyrophosphate, a powerful inhibitor of mineralization, to inorganic phosphate, which facilitates mineralization. In humans, there are four isozymes: germ cell ALP, placental ALP, intestinal ALP, and tissue-nonspecific ALP (bone/liver/kidney ALP).

Bone-specific ALP (BALP) regulates bone mineralization and is anchored to osteoblasts and matrix vesicles and can, in its circulating soluble form, be used as a biomarker of osteoblast activity and bone formation [89]. BALP is increased during skeletal growth, fracture recovery, and various bone and mineral metabolic disorders [90].

Osteocalcin, a non-collagenous protein of the bone matrix produced by osteoblasts, circulates in its intact form (1–49 amino acids) and as various fragments, which serve as surrogate markers for bone turnover. Osteocalcin controls blood glucose and fat deposits and, through osteocalcin, bone may therefore have a role in

energy metabolism [59]. High levels of urinary osteocalcin mirror bone turnover and are associated with short-term fracture risk in older women [91].

When type I collagen is formed in the bone matrix, propeptides of type I collagen are generated. The circulating levels of carboxy-terminal and amino-terminal propeptides thus reflect the synthesis of type I collagen.

Amino-terminal propeptide of type I procollagen (P1NP), derived from post-translational cleavage of type I procollagen, is an indicator of collagen formation that increases in bone formation-stimulating therapy [56].

Bone resorption markers

Bone resorption markers are produced as a result of osteoclast activity and as products of type I collagen degradation. Peptide fragments of type I collagen, such as *carboxy-terminal* and *amino-terminal crosslinked telopeptides of type I collagen* (CTX and NTX), are released into the blood when bone is resorbed. CTX and NTX are bone resorption markers with high specificity and sensitivity for the degradation of type I collagen.

Tartrate-resistant acid phosphatase isoform 5b (TRAcP 5b), produced by osteoclasts, is used as a marker of bone resorption, more specifically the number of osteoclasts [92]. The International Federation of Clinical Chemistry and the International Osteoporosis Foundation recommend P1NP and CTX as reference markers for bone turnover [93].

Table 3. Markers of bone turnover

Bone formation markers	**Bone resorption markers**
Bone-specific alkaline phosphatase (BALP)	Carboxy-terminal crosslinked telopeptides of type I collagen (CTX)
Osteocalcin	Amino-terminal crosslinked telopeptides of type I collagen (NTX)
Carboxy-terminal propeptide of type I procollagen	Tartrate-resistant acid phosphatase 5b (TRAcP5b)
Amino-terminal propeptide of type I procollagen (P1NP)	

2.3.6 SCLEROSTIN

Sclerostin is a protein encoded by the *SOST* gene that is predominantly produced by mature osteocytes. Through its inhibitory effect on the Wnt/β-catenin signaling pathway, sclerostin regulates bone formation. The Wnt/β-catenin signaling pathway controls differentiation of mesenchymal stem cells, inhibiting chondrogenic and adipogenic differentiation and favoring osteoblastic differentiation. This signaling pathway also promotes maturation of osteoblasts and survival of osteoblasts and osteocytes, and inhibits the generation of osteoclasts [94]. Activation of this pathway thus leads to increased bone formation and decreased bone resorption and, accordingly, to high aBMD, such as in sclerostin deficiency seen in sclerosteosis and van Buchem's disease. One of the functions of osteocytes is mechanotransduction by activating the Wnt/β-catenin signaling pathway, allowing the skeleton to adapt the bone structure according to biomechanical needs and sclerostin appears to serve as a regulator of bone mass formed in response to mechanical loading [95]. Besides acting on osteoblasts, sclerostin is also believed to promote formation and activity of osteoclasts [96]. Accordingly, sclerostin can regulate both osteoblasts and osteoclasts. Sclerostin also regulates adipogenesis in the bone marrow and one study demonstrated that a decrease of sclerostin decreases bone marrow adipose tissue [97].

2.3.7 VITAMIN D

Vitamin D is a unique hormone in the respect that it can be synthesized in the skin from exposure to sunlight and plays an important role for calcium homeostasis and bone mass acquisition during growth. Vitamin D from the skin or diet is hydroxylated in the liver to 25-hydroxyvitamin D (25(OH)D), which is further hydroxylated in the kidneys to its active form, 1,25-dihydroxyvitamin D ($1,25(OH)D_2$). The 25(OH)D level in serum is used to determine a person's vitamin D status. Both PTH and serum levels of calcium and phosphorus regulate the renal production of $1,25(OH)D_2$ [98], and $1,25(OH)D_2$ in turn stimulates the reabsorption of calcium in the kidneys and the absorption of calcium and phosphorus in the intestine. $1,25(OH)D_2$ also interacts with the vitamin D receptor in osteoblasts, which finally results in inducing immature monocytes to become mature osteoclasts that mobilize calcium and other minerals from the skeleton (figure 6). IGF-I is also involved in vitamin D metabolism, by increasing the renal conversion of 25(OH)D to $1,25(OH)\ D_2$ [10, 11]. Studies have found that in obese individuals, an enhanced uptake of vitamin D metabolites by adipose tissue reduces the circulating levels [99, 100]. Some studies suggest that 25(OH)D

levels are associated with both high and low BMI, with an inverted u-shaped relationship between vitamin D and fat mass [101, 102].

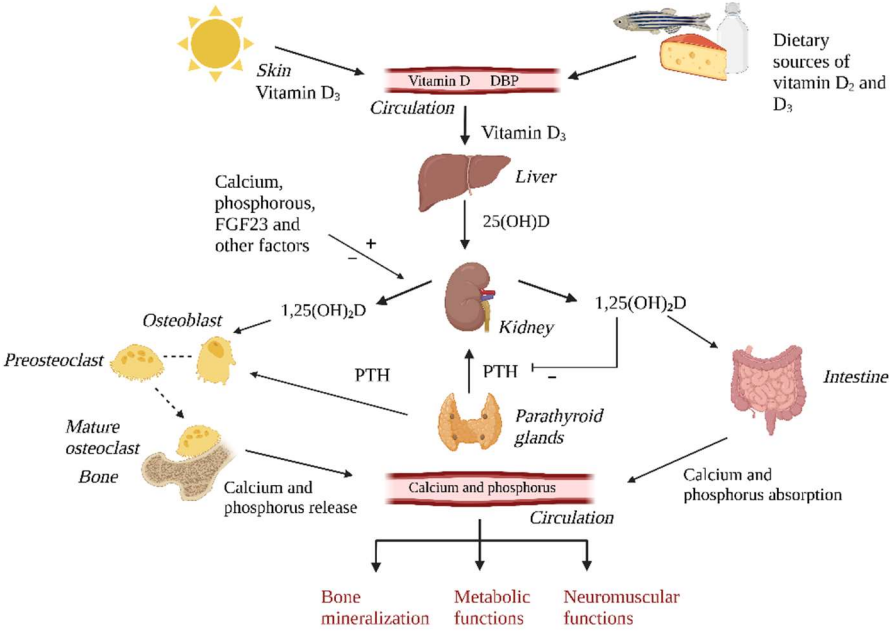


Figure 6. Vitamin D metabolism. Created with BioRender.com.

Vitamin D deficiency

Vitamin D deficiency results in decreased absorption of dietary calcium and phosphorus and an increase in PTH levels [98]. Secondary hyperparathyroidism maintains normal serum calcium levels by mobilizing calcium from bone and increasing the wasting of phosphorus in the kidneys. Accordingly, serum phosphorus levels decrease and cause a mineralization defect in the skeleton. PTH also mediates increased osteoclastic activity, leading to bone weakness and decreases aBMD resulting in rickets or osteopenia [103].

The Endocrine Society has defined vitamin D deficiency as 25(OH)D <25 nmol/L, insufficiency as 25(OH)D 25–50 nmol/L, and vitamin D sufficiency as at least 75 nmol/L for maximum musculoskeletal health [103]. The US Institute of Medicine and the Endocrine Society [103, 104] recommend a daily vitamin D

intake of at least 400 IU for children 0–1 years, 600 IU for children 1–18 years, and 600 IU for adults up to age 70, with the goal of keeping the serum level of 25(OH)D >50 nmol/L. A daily vitamin D intake of 1000 IU in children and 1500-2000 IU in adults is, however, required to maintain vitamin D sufficiency (25(OH)D >75 nmol/L) [103].

Free 25(OH)D

The majority of vitamin D is bound to vitamin D binding protein (DBP) (approximately 88%) and to albumin (approximately 12%). Only about 0.03% of total 25(OH)D circulates free and unbound [105]. DBP thereby serves as a circulating reservoir for vitamin D metabolites and seems to inhibit some of the actions of vitamin D [106]. In most subjects, free 25(OH)D is highly correlated to 25(OH)D and negatively associated to PTH [107]. Diseases that affect the synthesis of DBP or albumin may, however, affect the amount of circulating 25(OH)D. Patients with impaired liver function have reduced total 25(OH)D levels, but normal or increased levels of free 25(OH)D [108]. Estrogen stimulates DBP synthesis, and during pregnancy the affinity of DBP to vitamin D metabolites appears to be decreased, resulting in higher concentrations of free 25(OH)D during pregnancy [107]. In patients with low concentrations of DBP, such as malnourished patients, free 25(OH)D is more strongly associated with bone parameters than total 25(OH)D [109, 110]. Bacha et al. [111] demonstrated that in healthy adolescents, DBP is related to body fat mass but both total and free 25(OH)D vary in the same direction, inversely to DBP levels; hence, the use of free 25(OH)D rather than total 25(OH)D to evaluate vitamin D status was not supported. Serum-free 25(OH)D concentrations have been reported to constitute 0.02% to 0.09% of total 25(OH)D concentrations [105], with a normal range of 1.25 to 20.25 pmol/L in healthy adults [112].

PTH

PTH is secreted from the parathyroid glands and regulates the serum calcium concentration by enhancing renal calcium absorption, increasing the activation of 25(OH)D to increase the intestinal calcium absorption, and mobilizing calcium from bone by increasing bone turnover [113]. PTH occurs in two forms: *non-oxidized PTH* (n-oxPTH) and *oxidized PTH* (oxPTH), and the traditional PTH assays detect both forms. n-oxPTH and oxPTH have different biological properties, and only n-oxPTH is able to regulate calcium and phosphate metabolism and activate the PTH receptor [114]. In patients exposed to oxidative stress (e.g. chronic kidney disease), a considerable amount of PTH is oxidized [115].

2.4 BONE MASS MEASUREMENTS

2.4.1 DUAL-ENERGY X-RAY ABSORPTIOMETRY

The DXA method was introduced in 1987 and has become the gold standard for clinical bone density measurement [116]. X-rays in two different energy levels are used to separate the mineralized and soft tissue components of the scanned site. DXA has several practical advantages, such as the short scanning time, the high availability, and the low dose of radiation, and is the preferred method for measuring children. Its primary limitation is that it is a two-dimensional method of measuring aBMD, thus providing a two-dimensional image of a three-dimensional structure. This aspect is important to consider when measuring children, in whom growth results in alterations in both size, shape, and density of the bones.

Figure 7. 5-year-old boy undergoing DXA measurement. Photo by Ann-Sofie Petersson. With permission from child and parents.

DXA provides an estimate of BMC (g), which is divided by the projected area of the bone (cm^2), giving aBMD (g/cm^2). Results are also presented as aBMD Z-scores (SD), which are calculated based on reference data from the US National Health and Nutrition Examination Survey (NHANES) and the manufacturer, based on age, gender, body weight, and ethnicity. During growth, BMC increases related to the increase in bone size, but vBMD (mg/cm^3) remains stable [19]. Accordingly, the aBMD Z-scores will be inaccurate in very short or very tall children. The recommendation from the ISCD 2013 [85] was to include lumbar spine and total body less head (TBLH) in pediatric DXA examinations. The spine is considered a preferred site because of the precision of measurements and the availability of large amounts of pediatric reference data. The rationale for

excluding the head in measurements of children is that the head makes up a large part of the total body bone mass and will probably not change as much as the rest of the bone mass because of disease or treatment or in response to environmental stimuli such as physical activity. Scans of total body also enable the evaluation of body composition (fat mass and lean mass). The ISCD further recommends adjusting spine and TBLH BMC and aBMD using bone mineral apparent density (BMAD) or the height Z-scores in children with short stature or growth delay [85]. A method has been developed to adjust LS BMD and LS BMAD for height and BMI in children and adolescents [117]. Since bone mass is greatly influenced by sex steroids, it is also important to consider bone age and pubertal status when measuring children with chronic diseases, which are frequently associated with impaired growth and sexual maturation [118]. In the updated guidelines from ISCD 2019 [119], it is stated that there is now sufficient data to recommend other sites for DXA measurements in children, such as distal forearm, proximal and lateral distal femur, and vertebral fracture assessments. This can be useful in children where TBLH or lumbar spine scans are not feasible because of orthopedic implants or devices (giving imaging artifacts), severe osteoporosis, or difficulties with positioning.

2.4.2 PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY

pQCT is a three-dimensional technique that measures vBMD independently of bone size, and pQCT measurements can therefore complement the information given by DXA. This method makes it possible to measure cortical and trabecular bone compartments separately and to assess geometric parameters that may contribute to bone strength, such as muscle and cortical area, and periosteal and endosteal circumference. The most commonly measured sites are the tibia and the radius. Limitations with this method are the lack of pediatric reference data and the difficulties in positioning the arm or leg of young children in the measurement gantry; therefore, the ISCD has stated that in children, this method should primarily be used for research [120]. A more recent development of the pQCT technique is high-resolution pQCT, which allows assessment of the architecture of cortical and trabecular bone [121].

2.4.3 DUAL-ENERGY X-RAY ABSORPTIOMETRY AND LASER

The dual-energy X-ray absorptiometry and laser (DXL) technique is a method combining DXA with laser measurement of the heel thickness of the foot. With the DXL technique, aBMD and BMC of calcaneus (consisting primarily of trabecular bone) can be measured with high accuracy. Advantages with this method are the very short scan time, the low radiation dose, and the fact that the device is portable. The DXL method predicts osteopenia well and is highly correlated with the whole body DXA method [122-124]. In the DXL Calscan pediatric version, manual measurement of the calcaneal height is possible, which, together with aBMD, enables calculation of BMAD (g/cm^3); this makes it possible to correct for different bone sizes, which is important when assessing bone mass in growing individuals [125].

2.5 ANOREXIA NERVOSA

AN is a psychiatric disorder characterized by low body weight and secondary conditions associated with malnutrition. The weight loss due to impaired energy balance (primarily reduced food intake, but also increased physical activity), deficits in specific dietary components and weight control, such as purgative behaviors, may together endanger health. Hence, AN is associated with both devastating mental health consequences and serious somatic consequences, such as osteoporosis, nutritional deficiencies, and cardiac and metabolic disorders, and mortality is high [126]. A systematic review reported weighted population means of lifetime prevalence of AN in women to 1.4% (range 0.1–3.6%) and for men 0.2% (range 0–0.3%) [127]. AN predominately develops in adolescence, in the peripubertal period, with up to 25% of girls with AN developing the disease before 13 years of age [128]. The prognosis is less favorable and the risk of chronic forms greater if eating disorders appear before puberty [126]. In recent years, the understanding of the underlying neurobiology has advanced, and it is today considered that genetic factors influence the risk of developing AN and that both intrapersonal and psychosocial factors may trigger the onset. This has led to a shift away from a simple sociocultural explanatory model towards one integrating biological and environmental factors [129].

The criteria for AN according to the Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM IV-TR) include limitation of voluntary food intake, weight loss, or failure to gain weight during a period of growth, resulting in a body weight less than 85% of that expected for age and height, intense fear of gaining weight even though underweight, disturbed perception of body weight or shape,

and primary or secondary amenorrhea [130]. In 2013, the criteria for AN was revised by the American Psychiatric Association in DSM-V [131] by making the weight criteria less stringent and by removing amenorrhea as a criterion, enabling the diagnosis of AN to be extended to boys and men, premenarcheal girls, women who continue to menstruate despite severe underweight, and women who are taking hormonal contraception.

Today treatment programs for adolescent patients with AN that involve the family early and actively are regarded as best practice [129, 132]. Patients with severe AN and patients who have failed to respond to outpatient care might require inpatient care. BMI <14 kg/m^2 is considered life-threatening and hospital admission may be required; however, it is also important to consider the rapidity of weight loss and mental health. BMI <11 kg/m^2 is associated with a dramatically increased mortality risk [126]. The cornerstone of treatment for AN is nutritional restoration and the rate of weight restoration is a positive predictor of weight recovery one year after hospitalization [133]. Approximately 50% of women with AN recover after therapy [134, 135]. A Swedish long-term follow-up by Dobrescu et al. [136], found that 64% of women with AN had full recovery from eating disorder symptoms after 30 years and 6% still had a diagnosis of AN. Later onset of adolescent AN was associated with a better outcome.

2.5.1 NUTRITION TREATMENT

Refeeding is the first important step to recovery from AN, and a combination of psychotherapy and refeeding is recommended [137]. A multidisciplinary team is crucial, allowing psycho-behavioral therapy and guidance on energy needs and meal plans as well as supplementation with vitamins and minerals. The aims of refeeding are to improve both the nutritional and mental state of the patient, to manage acute somatic effects (such as hypoglycemia, hypotension, and bradycardia), and to reduce the risk of somatic complications such as growth failure, delayed or arrested puberty, and low bone mass [138].

The need for weight gain must be balanced against the risk of *refeeding syndrome*, which may occur as a result of increased insulin levels when nutrients are reintroduced after starvation, leading to movement of glucose, electrolytes, and liquid from the extracellular to the intracellular space. Metabolic disturbances (hypoglycemia, hypophosphatemia, hypocalcemia, hypokalemia, or hypomagnesemia) can occur and may lead to cardiac arrythmia or failure, seizures, coma, and sudden death [139]. The recommendation for refeeding in AN was for many years to "start low and go slow" to avoid refeeding syndrome [137].

However, with this protocol there was an initial weight loss and the following weight gain was slow [140]. This led to increasing interest in a more aggressive approach of refeeding in AN, and several studies with higher caloric intake from the start of treatment or more rapid advancement showed positive results without an increased frequency of refeeding syndrome [141, 142]. A review by Garber et al. [143] found that faster weight gain and higher weight upon discharge from hospital were predictive parameters for weight recovery after 1 year. Severely ill AN patients (BMI <15 kg/m^2) are at greater risk of refeeding syndrome and there is insufficient evidence for a more aggressive refeeding approach in these patients [143]. The updated guidelines from the American Psychiatric Association suggest individually determined target weights, where one estimate of target weight is the weight at which reproductive function normalizes. Typically, menses will resume at 90–95% of median BMI, which corresponds to a body fat percentage of approximately 21%. A BMI of 20 kg/m^2 can also be used when determining a target weight [144]. The recommendation from the Swedish Psychiatric Association from 2015 [145] is an energy intake of 3000 kcal/day to achieve weight gain, but a lower energy intake of 20–30 kcal/kg/day is recommended from treatment start in severely ill AN patients, which is then increased by 200–300 kcal every third day. The recommended weight gain is 0.5 to 1.0 kg/week. However, personalized goals for target weight, determined from previous charts, are recommended. The use of prophylactic phosphate supplementation varies; in some studies it is used for patients with BMI <12 kg/m^2, in other studies it is used in nasogastric feeding but not meals-only feeding [143]. Since the small increase in weight gain with supplemental total parenteral nutrition does not outweigh the risk, the only indication for total parenteral nutrition in AN is when there are no other alternatives [143].

2.5.2 ENDOCRINE DYSREGULATION IN ANOREXIA NERVOSA

The function of the hypothalamic-pituitary-ovarian axis is dependent on energy availability, and the malnutrition in AN is associated with central suppression of this axis, resulting in low luteinizing hormone pulses from the pituitary gland, and finally decreased estrogen secretion from the ovaries [146]. Boyar et al. [147] found an immature luteinizing hormone pattern in AN girls, that resembled that found in prepubertal girls; it matured if their weight returned to normal, indicating the reversibility of this abnormality. Since leptin simulates gonadotropin releasing hormone secretion, the reduced fat mass and consequent hypoleptinemia in AN might contribute to this state [148]. There is, however, no clear association between body weight or fat mass and amenorrhea, and in up to 15% of women

with AN, amenorrhea persists despite recovery of normal body weight [149]. With increasing knowledge about the endocrine dysregulation in AN and the observation that some women with AN and low weight continue to have regular menses, amenorrhea was removed as a criterion for AN in DSM-V [131].

AN during puberty can result in growth stunting, related to the lack of energy, deficiency in nutrients such as calcium and vitamin D, estrogen deficiency, and reduction of IGF-I secretion. GH concentrations are increased in AN as a result of nutritional deprivation, with the highest levels seen in patients with the lowest BMI and fat mass [150]. Due to resistance to GH in AN, the secretion of IGF-I is reduced, and the negative feedback decreased. Low levels of IGF-I might be an adaptive response to preserve energy during chronic starvation by decreasing energy expenditure on growth. Refeeding increases the levels of IGF-I [151] and GH secretion normalizes with weight gain [152].

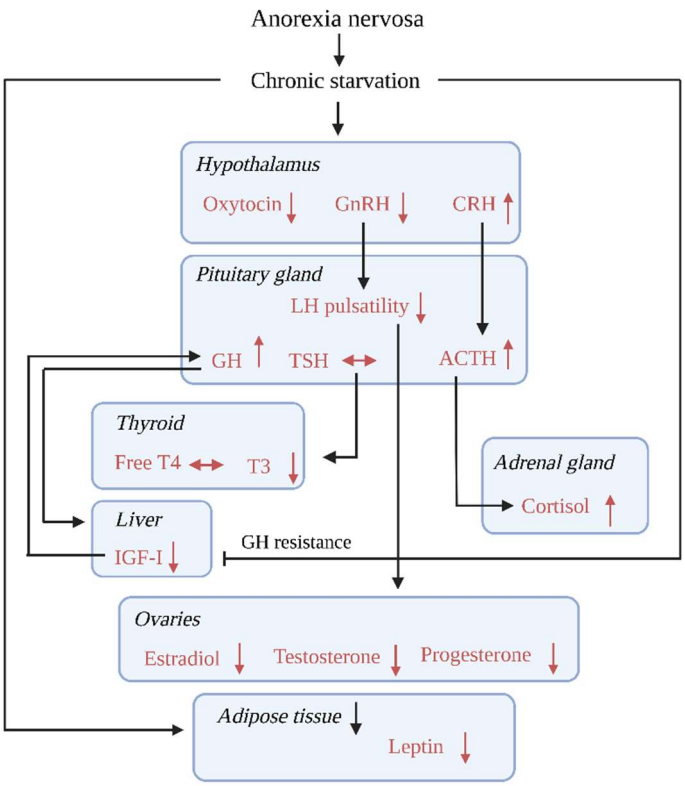


Figure 8. Endocrine dysregulation in AN. Created with BioRender.com.

2.5.3 BONE HEALTH IN ANOREXIA NERVOSA

Through its inhibitory effect on osteoclasts and stimulatory effect on osteoblasts, estrogen plays an important role in bone remodeling, and estrogen deficiency in amenorrheic women with AN is a key factor mediating AN-related bone disease [153]. The estrogen deficiency and undernutrition in female adolescents with AN, during a period when physiologic estrogen secretion is necessary for optimizing bone acquisition, may thus result in diminished acquisition of PBM. There is a well-established association between the duration of AN and early onset of amenorrhea (indicating a longer duration and severity of estrogen deficiency) and lower aBMD [154]. The cause of bone loss in AN might also be related to other hormonal abnormalities such as low leptin, low testosterone concentrations, high cortisol concentrations, reduced IGF-I expression, GH resistance, and low dehydroepiandrosterone concentrations (figure 8) [153, 155]. Soyka et al. [156] reported that the duration of AN was the most significant predictor of spinal aBMD and further that both estrogen and testosterone, as well as IGF-I and bone formation markers, were reduced in female AN patients. In another study, decreased IGF-I concentrations were negatively associated with aBMD and microarchitectural parameters, independent of BMI [157]. Hypercortisolemia, a result of the stress of chronic nutritional deprivation aiming at maintaining euglycemia, is also related to a reduction in bone formation markers and inversely related to aBMD [158]. Consequently, patients with AN may achieve a lower PBM and later in life more quickly reach osteoporotic levels.

One study found that aBMD at one or more skeletal sites was reduced by at least 1.0 SD in 92% of AN patients and by at least 2.5 SD in 38% (mean age 24.4 years, mean duration of AN 66 months) [159]. Lawson et al. [157] demonstrated abnormal trabecular microarchitecture in women with AN, and that this microarchitecture correlated to aBMD at multiple sites as well as to IGF-I, leptin, and androgen levels. Results for bone turnover markers in AN vary, with some studies demonstrating decreased bone formation and increased bone resorption [160, 161] and other studies pointing at a decrease in both bone formation and resorption [162, 163].

The age of onset of AN is a crucial parameter, and the impact on bone is greater when the onset is at a younger age. Castro et al. [164] evaluated the catch-up effect in aBMD in adolescent girls with AN and found that patients with a poor short-term outcome (defined as BMI <19 kg/m^2 and amenorrhea) showed a further decrease in aBMD at follow-up (mean 15 months), whereas patients with a good short-term outcome (BMI >19 kg/m^2 and regular menses) had a significant gain in aBMD per year. The conclusions from this study were that weight-recovered AN patients can reach normal values of aBMD in the long term. In contrast to

this, a long-term follow-up found lower hip aBMD in women 21 years after recovery from AN, than in healthy controls [165].

Adequate 25(OH)D levels are a prerequisite for bone health in AN. A meta-analysis by Veronese et al. [102] found that, without supplementation, patients with AN had lower levels of 25(OH)D than healthy controls. With supplementation, however, 25(OH)D levels were higher in AN patients than controls, which was interpreted as likely explained by the lower fat mass.

Giollo et al. [166] studied female patients with AN (13–45 years) before and after intensive weight-restoration treatment and found that weight restoration was associated with a rise in spine aBMD, but the association was only detectable in patients with 25(OH)D ≥75 nmol/L, which led to their strong recommendation to supplement AN patients with vitamin D. The Endocrine Society supports maintaining vitamin D levels higher than or equal to 75 nmol/L in patients with AN to favor optimal bone health [103], and the preferred range of 25(OH)D is 100–150 nmol/L [167].

2.6 THE MODIFIED ATKINS DIET

Up to 65% of individuals with epilepsy respond to anti-seizure drugs or enter spontaneous remission [168]. However, a large proportion of patients have refractory epilepsy and their chance of responding to medication falls dramatically after failure to respond to more than one drug [169]. For children with refractory epilepsy, treatment options include vagal nerve stimulation, epilepsy surgery, and the ketogenic diet (KD). Prior to the development of the KD, the use of fasting as a treatment for epilepsy has been described since at least 500 BC. In 1921, it was observed that starvation or consuming a high fat and very low carbohydrate diet resulted in the formation of ketones. In the 1920s and 1930s, the KD was widely used as a treatment for epilepsy, but usage decreased as new and more effective anti-seizure drugs became available; however, the KD regained popularity during the 1990s.

The term KD refers to any diet therapy resulting in a ketogenic state of metabolism, usually a low carbohydrate, moderate protein, and high fat diet. In this state, fatty acids are used to produce ketone bodies (acetoacetate and beta-hydroxybutyrate) that replace glucose as the major energy source for the brain. The ketone bodies can penetrate the blood–brain barrier and exert an anticonvulsant effect through several mechanisms, including reduced neuronal

excitability and changes in cellular metabolism [170], elevated levels of gamma-aminobutyric acid, and stabilized synaptic function [171].

Initiation of classic KD requires inpatient hospitalization for 1 to 3 days. A majority of patients on KD achieves seizure reduction, but previous studies indicate that KD treatment may lead side effects such as alteration in body composition, growth failure, osteopenia, kidney stones, and metabolic acidosis [172-175]. The frequency and severity of side effects appear to increase with the restrictiveness of the diet [176].

The MAD is a variant of KD created at John Hopkins Hospital in 2003; it is available as a treatment for children with refractory epilepsy for whom epilepsy surgery is not an option, and for metabolic conditions where the brain and mitochondria are incapable of using glucose for energy metabolism, such as pyruvate dehydrogenase complex deficiency (PDHCD) and glucose transporter type 1 deficiency syndrome (GLUT1-DS). The MAD is less restrictive than the classic KD and can be started in an outpatient setting. With this diet, calories are never restricted and the protein intake is not limited; hence, this diet is preferable for patients having difficulties in tolerating the restrictive KD and for adolescents and adults with a larger appetite. Common side effects with MAD are fatigue, hypoglycemia, metabolic acidosis, constipation, nausea, and gastroesophageal reflux. More unusual side effects are hyperlipidemia, kidney stones, pancreatitis, and cardiomyopathy. Contraindications are porphyria and metabolic conditions where the breakdown of fat is impaired, or where the body is dependent on glycolysis. If feeding difficulties or vomiting exist, percutaneous gastrostomy is recommended. Several studies have reported efficacy of MAD in seizure reduction, with at least 30% of patients having >50% seizure reduction [177-180], and no inferiority to the KD [181]. One meta-analysis demonstrated that classic KD was superior to MAD in achieving >50% seizure frequency reduction, but that after 6 months, both diets showed a seizure frequency reduction >90% and seizure freedom was comparable between the diets [182]. El-Rashidy et al. [183] reported that 13% of patients on MAD dropped out because of diet intolerance and weight loss. In contrast to this, Kossoff et al. [177] found that weight and BMI were not affected after 6 months on MAD in children. Patients with therapy-resistant epilepsy who respond well to the diet are usually treated for 2–3 years, whereas children with GLUT1-DS or PDHCD are commonly treated throughout childhood. Responders to the MAD have a good chance of reducing the number of anti-seizure drugs needed.

3 AIMS

The general aim of this thesis was to investigate the effects of nutrition treatments on bone health and body composition in two cohorts; young women with nutrition treatment for severe AN, and children treated with MAD for intractable epilepsy or neurometabolic conditions.

The specific aims of each study were:

I. To evaluate vitamin D status and its association to BMI, fat mass, and bone mass in patients with AN during intensive nutrition treatment.

II. To study the potential relationship of the *FTO* gene to BMI and body composition in young women with AN during 12 weeks of intensive nutrition treatment.

III. To evaluate bone mass, bone turnover markers, and body composition in young women with AN three years after intensive nutrition treatment compared to a healthy comparison group.

IV. To prospectively assess bone mass, growth, and body composition in children treated with MAD for 24 months.

In the papers in this thesis, the terms "nutrition treatment" and "nutrition therapy" are used interchangeably.

4 HYPOTHESES

I. We hypothesized that malnutrition in severe AN is associated with vitamin D deficiency and that the intensive nutrition treatment would lead to improved vitamin D status in these patients, favorable for bone acquisition.

II. Our hypothesis was that the individual *FTO* genotype may be associated with the increase in body weight and fat mass during nutrition treatment, and possibly constitute a basis for individualized nutrition treatment in AN patients.

III. We hypothesized that intensive nutrition treatment in women with severe AN would lead to maintenance of normal BMI after 3 years, and consequently improvement of bone mass and biomarkers reflecting bone turnover after 3 years.

IV. Our hypothesis was that treatment with MAD for 24 months would, as previously described with KD, involve negative effects on linear growth and bone mass in children with refractory epilepsy and neurometabolic conditions.

5 PATIENTS AND METHODS

5.1 STUDY SUBJECTS

5.1.1 PAPERS I-III

Papers I and II are based on a cohort of young women (aged 16.0–24.2 years) with severe AN who were admitted to the Child and Adolescent Psychiatry department at the Queen Silvia Children's Hospital, Gothenburg, Sweden, for a planned inpatient treatment with 12 weeks' duration. All patients who met the inclusion criteria: female, age 16–24 years and a diagnosis of AN (DSM-IV), were invited to participate. The exclusion criteria were: inflammatory bowel disease, diabetes mellitus, and medication affecting the bone, such as glucocorticoids. From January 2012 to July 2014, 27 individuals were asked to participate and 25 accepted. Twenty-two women underwent the 12-week nutrition treatment.

Paper III is a prospective follow-up study of the same cohort of AN individuals. Approximately three years after the nutrition treatment, between august 2015 and July 2017, all 22 individuals were contacted and asked to participate in the follow-up study, twenty women accepted participation. A group of 17 age-matched healthy women was randomly recruited among hospital staff and students at Gothenburg university and included between November 2017 and January 2018. A flow-chart of papers I-III is presented in figure 9.

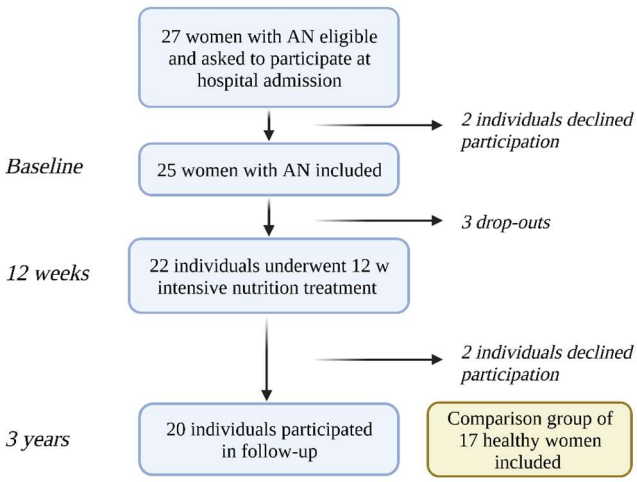


Figure 9. Flow chart of young women with AN examined in papers I–III. Created with BioRender.com.

5.1.2 PAPER IV

Paper IV was a prospective cohort study of 38 pediatric patients (21 girls, 17 boys) treated with MAD at the Department of Neurology, Queen Silvia children's hospital, Gothenburg, Sweden, between 2010 and 2017. The inclusion criteria were disease onset before 18 years of age and a treatment period with MAD of at least 6 months; there were no exclusion criteria. Thirty patients had seizures prior to study start; median seizure frequency was 200 seizures/month (1–1532). The median age at initiation of MAD treatment was 4.4 years and the median duration of epilepsy 3.0 years. Twenty-nine patients continued MAD treatment for 24 months. Several etiologies were present in this cohort (table 4).

Table 4. Etiologies among 38 children treated with MAD in paper IV

PDHCD	9 (23.7%)
GLUT1-DS	7 (18.4%)
Genetic epilepsy	7 (18.4%)
Epilepsy of unknown etiology	4 (10.5%)
Cortical malformation	3 (7.9%)
Other mitochondriopathies	2 (5.3%)
Tuberous sclerosis	2 (5.3%)
Encephalitis	2 (5.3%)
Stroke	1 (2.6%)
Aicardi syndrome	1 (2.6%)

(GLUT1-DS = glucose transporter type 1 deficiency syndrome,
PDHCD = pyruvate dehydrogenase complex deficiency.)

5.2 NUTRITION TREATMENTS

5.2.1 INTENSIVE NUTRITION TREATMENT

At the time of the study reported in papers I and II (2012–2014), the eating disorder unit at the Child and Adolescent Psychiatry Department at the Queen Silvia Children's Hospital, Sahlgrenska University Hospital, Gothenburg, practiced a 12-week intensive nutrition treatment as inpatient care for patients with severe AN [184]. This treatment protocol, introduced around 2005, consisted of a high energy diet (>3000 kcal/day) from start, and patients were offered a great variety of foods, with the aim of both restoring body weight and changing the patients' attitudes to food, which in turn aimed at normalizing eating behavior. Energy intake was gradually decreased as the patients gained weight, with the aim of obtaining energy balance. This treatment protocol differed from those commonly used at the time of this study, where the energy intake was instead gradually increased.

Patients were included in the study when admitted to the eating disorder unit for 12 weeks of inpatient treatment. The menu from day 1 consisted of six meals (breakfast, lunch, dinner, night meal, and two snacks) and was planned by an experienced dietician. From day 2, all patients were also served liquid nutritional supplements (3 x 200 ml, 1.5 kcal/ml). All patients were fed orally, with no nasogastric feeding. When the patients gained weight, the liquid supplements were gradually removed from the menu; hence, the total energy intake decreased over 12 weeks (from median 3351 kcal/day, 75 kcal/kg/day the first week to median 2518 kcal/day, 46 kcal/kg/day the last week). The nutrient intake was calculated based on the Nordic Nutrition Recommendations [8], with a fat content of 34 percent of total energy (E%) and protein content 17 E% [184]. The mean daily intake of vitamin D was 15 μg (600 IE) and calcium 2120 mg through the diet, and all except one patient were in addition given daily supplements of 10–20 μg (400–800 IE) vitamin D and 500–1000 mg calcium. In the beginning of the treatment, patients were supported to rest and relax between meals. Later, patients were encouraged to take part in low-intensity group activities and to take short and slow walks. Every week, the patients met a psychologist for individual and/or family therapy sessions. In addition, a discussion group focusing on psycho-education was offered every week. Overall, the treatment protocol was staff intensive, with 24-hour surveillance.

Body weight was measured every week in the morning hours before breakfast during the period of nutrition treatment, and after 3 years. Height was measured once before start of treatment and after 3 years. Blood samples were collected, and bone mass measurements were performed on day 1, after 12 weeks, and after

3 years. At baseline, the patients answered a questionnaire about duration of AN, amenorrhea, vitamin D and calcium supplementation, medication, fractures, family history of osteoporosis, and physical activity. At the 3-year follow-up, patients answered a questionnaire about treatment for AN during the follow-up period, amenorrhea, vitamin D and calcium supplementation, and fractures, with a separate questionnaire about physical activity (IPAQ [185]).

5.2.2 THE MODIFIED ATKINS DIET

The diets for all patients were calculated by the same hospital dietician, and all children started with 10–30 g carbohydrates per day. The child's food preferences were taken into account, and the initial calorie content was based on an average of the intake before initiating MAD treatment. Based on the content of every child's diet and according to the Nordic Nutrition Recommendations [8], subjects were supplemented with vitamins and minerals (vitamin D, calcium, phosphorus, magnesium, carnitine, and potassium citrate). There was no limit to the amount of protein, fat, or fluids. The KD ratio (ratio of fat to protein + carbohydrates) was between 2:1 and 4:1 in all patients. During the treatment, the diet and KD ratio were adjusted based on the effect on seizures, level of ketosis, weight, and wellbeing. As in normal clinical routine, the treatment effect was evaluated after 3–6 months.

During the first week of the MAD treatment, the children were partly hospitalized. Blood and urine samples were collected daily, as a part of clinical routine, to evaluate the effects and to avoid side effects. Weight, height, and blood samples were measured before start of MAD, and after 6, 12, and 24 months. Seizure frequency was documented by the family before start of MAD treatment and thereafter during the month before every follow-up visit. DXA and/or DXL measurements were performed at baseline and after 12 and 24 months.

5.3 AUXOLOGICAL MEASUREMENTS

In papers I–III, body weight was measured without heavy clothes and shoes to the nearest 0.1 kg on the same calibrated weight scale before breakfast prior to the start of nutrition treatment, then weekly during the 12-week treatment period, and after 3 years. Height was measured to the nearest 0.5 cm without shoes with a wall-mounted ruler and BMI was calculated as weight/height^2 (kg/m^2).

In paper IV, body weight and height were measured at baseline and after 6, 12, and 24 months. The height measurements were taken three times and the mean value was calculated. Height SDS and BMI SDS were calculated according to the Swedish growth reference from birth to 18 years [7, 186].

5.4 BONE MASS MEASUREMENTS

5.4.1 DUAL-ENERGY X-RAY ABSORPTIOMETRY

In papers I–IV, two-dimensional bone mass (BMC and aBMD) was measured with DXA for total body, lumbar spine (L1–L4), and total hip. Fat mass and lean mass were also assessed. The same skilled nurse performed all DXA measurements, and a daily calibration was carried out using a phantom provided by the manufacturer. Z-scores were generated automatically from reference data provided by the manufacturer.

Papers I and II: Lunar Prodigy (GE Lunar Corp., Madison WI, USA) was used for measurements in all AN patients at baseline and after 12 weeks. The coefficient of variation (CV) evaluated in 20 patients after repositioning was 0.5% for total body aBMD, 0.7% for lumbar spine aBMD, 2.4% for lean mass, and 0.9% for fat mass [187].

Paper III: After 3 years, 10 of the women with AN in papers I and II were measured with Lunar Prodigy, while the remaining 10 who attended the follow-up, as well as all of the comparison group (n=17), were measured with Lunar iDXA (GE Lunar Corp). The reliability between Lunar Prodigy and Lunar iDXA was evaluated and found to be acceptable (paper IV, Table 2). The DXA scans performed on Lunar Prodigy were reanalyzed with Lunar iDXA (enhanced analyses) after recommendation from the manufacturer.

Paper IV: Measurements of 31 children were performed on Lunar Prodigy, and two children were measured with Lunar iDXA. With Lunar iDXA, TBLH aBMD

and BMC are measured in patients under 20 years of age and both TB BMC and aBMD and TBLH BMC and aBMD are reported.

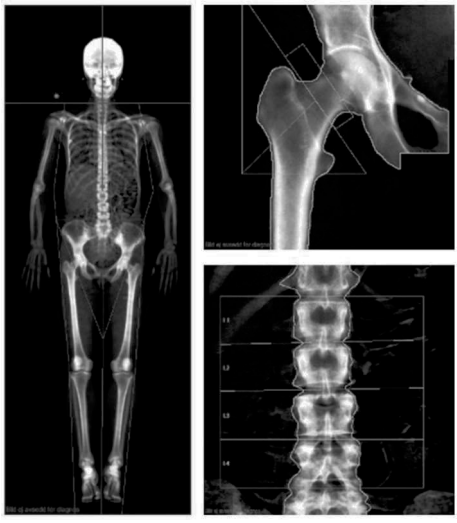


Figure 10. DXA scan of total body, hip, and lumbar spine. With permission from the patient.

5.4.2 PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY

In papers II and III, pQCT measurements were performed using XCT 2000 (Stratec Medizintechnik GmbH, Pforzheim, Germany) with software version 6.00 on the left tibia at 4% and 66% of the tibia length. A quality control was performed before each measurement using a standard phantom, and every 30 days using a cone phantom. The phantoms were provided by the manufacturer. All pQCT measurements were performed and analyzed by the same staff. The tibia length was measured manually from the medial malleolus to the medial tibia plateau. Correct position of the CT scans (the reference point) was defined in a coronal scan at the foot ankle and from this point, the 4 and 66% level of the tibia length were determined. A threshold of 180 mg/cm^3 was used at the 4% level to separate bone from soft tissue, and at the 66% level a threshold of 710 mg/cm^3 was used to separate soft tissue and bone marrow from cortical bone. All voxels with a density between 40 and 180 mg/cm^3 were assumed to be muscle. The intra-individual CV was 1.3% for total area, 0.4% for cortical density, and 0.8% for trabecular density [188].

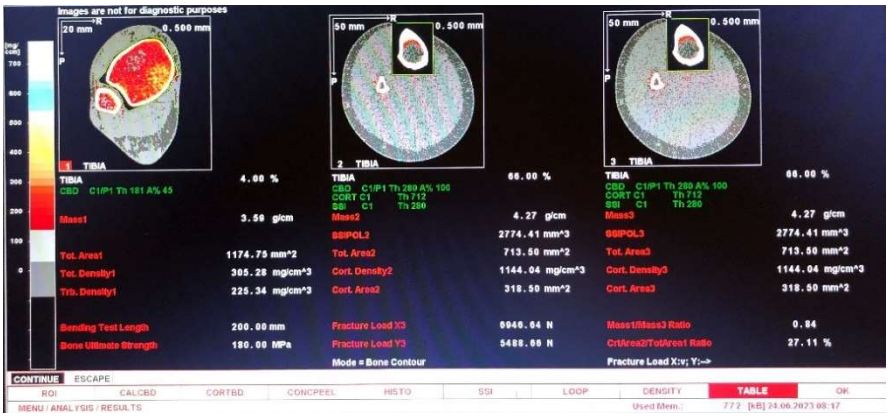


Figure 11. pQCT scan with cross-sectional analysis at 4 and 66% of the tibia length.

5.4.3 DUAL-ENERGY X-RAY ABSORPTIOMETRY AND LASER

The DXL Calscan system (Demetech AB, Täby, Sweden) was used to measure left calcaneal BMC and aBMD. The region of interest, with a fixed area of 0.74 cm^2, was manually placed in the trabecular part of calcaneus. The height of calcaneus was measured between the two processus tuberi calcanei. Bone mineral apparent density (BMAD; mg/cm^3) was automatically generated by dividing the aBMD by the calcaneal height. The interoperator CV for aBMD was 5.3% [125]. Intraindividual CVs for BMC and aBMD decreased with age (for 2-year-olds 9.8 and 10.1%, and for 7-year-olds 2.4 and 2.6%, respectively) [125]. DXL measurements are included in paper IV. DXL measurements were also performed in the 3-year follow-up of women with AN; the data are presented in the Results chapter, section 6.1.3.

A summary of the bone mass measurement methods used in this thesis is presented in table 5.

Table 5. Comparison of different bone mass measurement techniques

	Site **Bone parameters**	**Examination time (min)**	**Radiation dose (μSv)**	**Precision (CV%)**	**Advantages**	**Disadvantages**
DXA	Total body TBLH Lumbar spine Proximal femur BMC (g) aBMD (g/cm^2)	15	Lunar Prodigy: 1.55 iDXA: 14.24	Lunar Prodigy: 0.5–2.4 [189]	- High precision - Pediatric reference data from 5 years - Relatively short scan time - Measurement of body composition	- Integrated measurement of cortical and trabecular bone - Results dependent on height, bone age, and pubertal status - Irradiation, albeit a low dose
pQCT	Tibia Radius BMC (g) vBMD (g/cm^3) Periosteal and endosteal circumference (mm)	15	0.72	0.4–1.3 [188]	- Three-dimensional method - Results are independent of bone size - Trabecular and cortical bone measured separately - Measurement of muscle, fat, and geometric parameters	- Only peripheral sites can be measured - Long scan time - Sensitive to movement
DXL	Calcaneus BMC (g) aBMD (g/cm^2) BMAD (mg/cm^3)	<5	<0.12 (children) <0.2 (adults)	1.8–10.7 [190]	- Possible to assess area with predominantly trabecular bone - Low radiation dose - Short scan time - Portable machine	- Only calcaneus can be measured - Size dependent - Few studies with pediatric reference data

5.5 LABORATORY ASESSMENTS

In paper I, serum 25(OH)D and PTH were measured with electrochemiluminescence immunoassay (Roche Diagnostics Scandinavia AB, Gothenburg, Sweden) with intra- and inter-assay CVs <7%. The analytical range for 25(OH)D was 7.5–175 nmol/L and for PTH 0.5–535 pmol/L. Routine analyses in papers I and II were conducted at the Department of Clinical Chemistry, Sahlgrenska University Hospital, Gothenburg (accredited testing laboratory, SWEDAC no. 1240).

In paper II, the *FTO* gene was analyzed by DNA extraction from whole blood by the EZ1 BioRobot (Qiagen, Hilden, Germany). The *FTO* SNP rs9939609 was genotyped by polymerase chain reaction (PCR and then pyrosequencing). For details, see paper II.

In paper III, free 25(OH)D was analyzed by ELISA with intra-assay CV 5.5% and inter-assay CV 6.3%, analytical range 3.8–88.0 pmol/L (DIAsource ImmunoAssays SA, Louvain-la-Neuve, Belgium). Serum n-oxPTH was determined with affinity chromatography columns, where oxidized PTH binds to the column and n-oxPTH can be quantified (Immunodiagnostik AG, Bernsheim, Germany). BALP was measured using chemiluminescence immunoassay (CLIA) with intra-assay CV of 7.0% and inter-assay CV of 7.0%, analytical range 1.5–120 µg/L (DiaSorin Inc, Stillwater, MN, USA). Osteocalcin was measured by CLIA with analytical range 2.0–200 ng/mL, intra-assay CV 3.2%, and inter-assay CV 4.8% (Immunodiagnostic Systems Ltd, Boldon, UK). CTX was analyzed by CLIA with an analytical range of 0.03–6.00 ng/mL, intra-assay CV 3.5%, and inter-assay CV 5.9% (Immunodiagnostic Systems). Sclerostin was analyzed by enzyme-linked immunosorbent assay (Biomedica, Vienna, Austria) with intra-assay CV <7.0% and inter-assay CV <10.0%, analytical range 3.2–240 pmol/L. Leptin concentrations were determined by radioimmunoassay with intra-assay CV <7.0% and inter-assay CV <9.6%, analytical range 0.3–100 µg/L (human leptin RIA kit, Linco Research Inc, St. Charles, MO, USA). For details, see paper III.

In paper IV, IGF-I and IGF-BP3 were analyzed at the Gothenburg Pediatric Growth Research Center Laboratory. IGF-I was determined by using an IGFBP-blocked radioimmunoassay with excess of IGF-II. For analysis of IGF-BP3, a specific radioimmunoassay was used. For IGF-I, intra-assay CV was 7–15% and inter-assay CV 8–25%, and for IGF-BP3, intra-assay CV was 7–9% and inter-assay CV 10–20%. The results were converted to SDS according to age, gender, and pubertal stage. Routine analyses were conducted at the SWEDAC-accredited laboratory of Clinical Chemistry at Sahlgrenska University Hospital and at the

SWEDAC-accredited laboratories in Halmstad, Trollhättan, Borås, Skövde, Jönköping, and Karlstad.

5.6 STATISTICAL METHODS

In paper I, the Wilcoxon signed-rank test was used for tests of changes in continuous variables. Spearman's rank-order correlation coefficient, rs, and the corresponding *p* value were used to describe relationships between two continuous variables. All tests were two-tailed and conducted at the 0.05 significance level.

In paper II, Fisher's non-parametric permutation test was used to test for changes in continuous variables within a group and to test for differences between the groups. Fisher's non-parametric permutation test for pairwise comparisons was used for associations between continuous variables and ordered categorical variables.

In paper III, both Fisher's non-parametric permutation test for paired observations and Wilcoxon signed-rank test were used for comparisons over time. Fisher's non-parametric permutation test and Mann-Whitney U test and were used for comparisons between groups. To adjust for differences in BMI, when comparing the AN group and the comparison group at 3 years, ANCOVA was used.

In paper IV, Wilcoxon signed-rank test was used for changes in continuous variables over time within a group. For description of relations between two continuous variables, Spearman's correlation coefficient, rs, and the corresponding *p* value were used. All tests were two-tailed and conducted at the 0.05 significance level. Fisher' exact test and chi square exact test were used for the dropout analysis. A reliability study between Lunar Prodigy and Lunar iDXA was performed, the Bland Altman method was used to test for methodological differences between the DXA and the iDXA methods (paper IV, Table 2).

SAS Software version 9.3 and 9.4 (SAS Institute Inc., Cary, NC, USA) and IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA) were used for the statistical tests of papers I–IV. For all studies, statisticians from "Statistiska konsultgruppen", Gothenburg, were consulted for statistical advice.

5.7 ETHICAL CONSIDERATIONS

All studies in this thesis were carried out in accordance with the Declaration of Helsinki and were approved by the Regional Ethical Review Board in Gothenburg, Sweden:

Papers I and II: 20 December 2011, approval no. 720-11

Paper III: 1 June 2015, approval no. 389-15

Paper IV: 13 April 2012, approval no. 798-11

All participants, and, if under 18 years, their caregivers gave their informed consent to participate. All studies included in this thesis are based on nutrition treatments used in the clinic, and all patients who started the treatment regime within a specified period were invited to participate. Consequently, all patients received the same treatment regardless of whether they consented to participation in the study or not. An overall purpose with all studies included in this thesis was to evaluate treatments that were already routine in the clinic, with the aim of improving the treatments for future patients.

The number of participants in all the studies was relatively low, and from a statistical perspective, it would of course have been preferrable to include more patients in the studies. However, this is related to both the low incidence of these medical conditions (severe AN and therapy-resistant epilepsy), the practical circumstances in terms of the capacity of the departments where these patients were cared for, and the time aspect, since inclusion of more patients would have extended the duration of the studies significantly. However, it is our strong belief that studies like these nevertheless contribute with important insights about specific patient groups and treatments and should still be carried out. One can even argue that a decision not to conduct studies in children and adolescents with rare diseases would be unethical.

In papers I–III, all participants were young women, which is a limitation, since AN also affects young men. However, since the prevalence of AN is significantly higher in women than in men, it would not have been possible to include enough men in this study design to get statistical power and generalizable results. The patients were severely ill young women admitted for inpatient nutrition treatment. One can discuss whether it is ethically correct to ask individuals in such a state of psychiatric illness to participate in research. However, we found it defensible since the study did not have any impact on the patients' care or treatment. All patients were offered the same treatment regardless of whether they consented to

participation or not. The participants were informed about their DXA results by a doctor shortly after the investigation, and patients with compromised bone health were referred to the osteoporosis department at Sahlgrenska University Hospital after discharge from the anorexia ward. Including a control group from start would have been preferable; however, it would not have been ethically defensible to give healthy women this high-energy diet for 12 weeks with the aim of gaining about 10 kg in body weight.

In paper IV, both girls and boys, aged 1 to 17 years, were included. All patients who started treatment with MAD and fulfilled the study criteria during a specific period were invited to participate. All patients had the same treatment regardless of whether they consented to participation in the study or not. Inclusion of a control group would have been favorable; however, MAD has documented efficacy and it would have been highly unethical to withdraw potential patients from this treatment. Furthermore, an adequate control group would have to be matched for diagnosis, age, gender, weight, height, and pubertal stage and would therefore be difficult to recruit. Since there is robust reference data both for growth parameters and bone mass in children (for DXA from 5 years) we considered that using this data would be sufficient.

6 RESULTS

6.1 INTENSIVE NUTRITION TREATMENT (PAPERS I–III)

6.1.1 VITAMIN D

Twenty-five women with AN were included while admitted for intensive nutrition treatment and 22 completed the 12-week treatment. Twelve of the patients (48%) had supplementation with vitamin D prior to study start. The daily intake of vitamin D and calcium from the nutrition diet was mean 15 μg and 2120 mg respectively, and in addition, 24 women (96%) received daily supplements of vitamin D and calcium during the treatment period. All patients managed to eat all meals themselves, without the need for nasogastric feeding, including the five patients with BMI <15 kg/m^2 at baseline. No patient presented symptoms of refeeding syndrome.

Median 25(OH)D was adequate at baseline, 84.4 nmol/L (min 21.6, max 158.0), and decreased during the treatment period to median 76.0 nmol/L (min 30.5, max 131.0). Accordingly, there was a significant increase in PTH from 2.3 pmol/L to 3.2 pmol/L. Serum calcium and phosphate were stable. One patient was vitamin D deficient (<25 nmol/L) at study start. After 12 weeks, no patients had vitamin D deficiency, but values were insufficient (<50 nmol/L) in three patients.

Total body BMC increased, but aBMD was stable for 12 weeks. There were no associations between 25(OH)D and BMI, total body BMC, or aBMD at baseline or after 12 weeks. Nor were there any correlations between the differences in 25(OH)D and BMI, total body BMC, or aBMD.

Results paper I

- Vitamin D status was adequate in severely ill AN patients.
- 25(OH)D decreased during nutrition treatment, but was still sufficient.
- No associations were demonstrated between vitamin D status and BMI or bone mass parameters during 12 weeks of nutrition treatment.

At 3-year follow-up, 25(OH)D was stable, median 80.3 nmol/L. One AN patient was vitamin D deficient, whereas 25(OH)D levels were insufficient in three patients (15%). Free 25(OH)D was stable during the 12 weeks of nutrition treatment (median 18.1 pmol/L to 18.7 pmol/L), but significantly decreased to 17.0 pmol/L after 3 years (p=0.0026). Serum 25(OH)D and free 25(OH)D in AN patients showed a positive correlation (figure 12A). PTH increased significantly after 3 years to 3.8 pmol/L and PTH values were negatively associated with the 25(OH)D values over 3 years. In paper III, n-oxPTH was stable during the period of nutrition treatment for 12 weeks (0.99 and 0.95 pmol/L) but increased after 3 years to 1.13 pmol/L (p<0.0001).

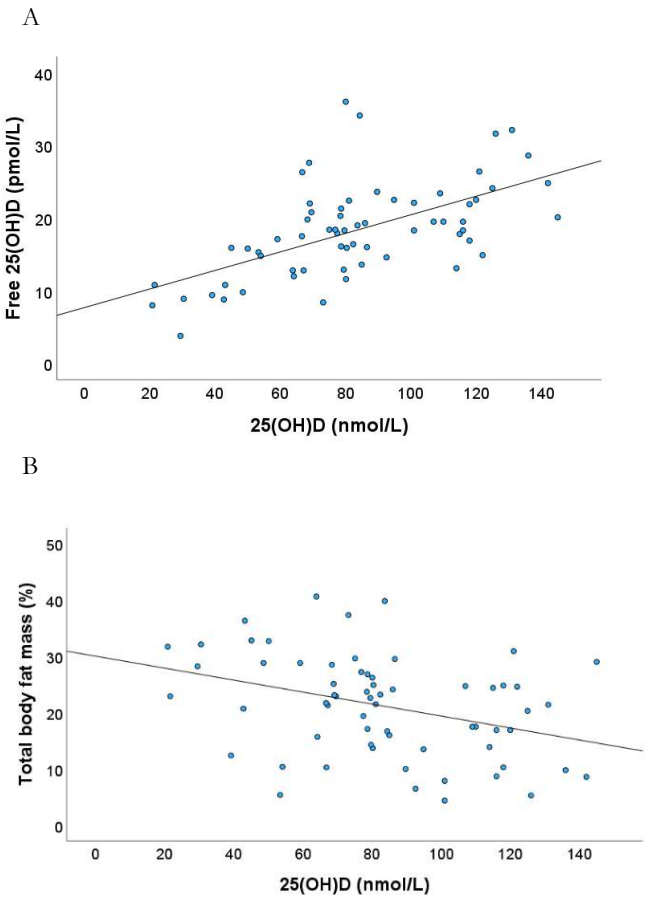


Figure 12. (A) Free 25(OH)D vs 25(OH)D in AN patients over 3 years, showing a positive correlation (r=0.608, p<0.001). (B) Fat mass vs 25(OH)D in AN patients over 3 years, showing a negative correlation (r=-0.368, p=0.003).

6.1.2 BODY COMPOSITION AND THE *FTO* GENE

The weight gain after 12 weeks of nutrition treatment was median 9.8 kg and BMI increased from 15.4 to 19.0 kg/m^2. Total fat mass increased by median 7.7 kg (median 12 to 27% of total body weight), including 4.2 kg increase in trunk fat mass (55% of the increase in total fat mass). The distribution of the *FTO* genotypes in AN patients was AA (homozygous for the risk allele) 24%, TA (heterozygous) 48%, and TT (homozygous for the non-risk allele) 28%, in accordance with the Hardy-Weinberg equilibrium [191]. All three genotype groups gained weight gradually during the treatment period. BMI and fat mass increased significantly in the TA and TT genotype groups while total lean mass was stable in all three genotype groups. The increase in weight or BMI during nutrition treatment did not differ between the genotype groups (paper II, page 5).

Results paper II

- There were no differences in changes in BMI or body composition during 12 weeks of intensive nutrition treatment between AN patients with *FTO* genotype AA, TA, or TT.

After discharge from hospital until follow-up after 3 years, a period with self-controlled energy intake, BMI, fat mass of the trunk and total body were stable in AN patients. Lean mass of trunk and total body as well as lean mass index were stable both during nutrition treatment and after 3 years but were significantly lower in AN patients than in controls. Body composition measured with DXA is presented in figure 13.

Fat area and muscle area, measured by pQCT, both increased during nutrition treatment but were then stable at 3-year follow-up. Fat area, but not muscle area, was lower in AN patients than the comparison group. In summary, the fat/muscle area ratio increased during nutrition treatment and then stabilized but was still lower than in comparison individuals after 3 years.

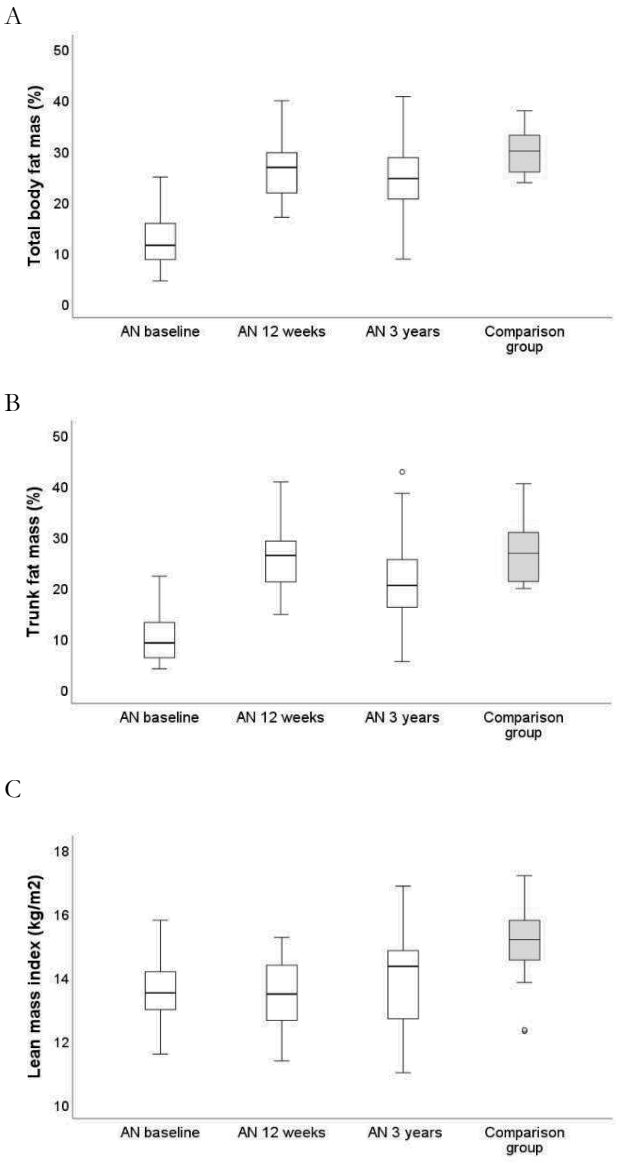


Figure 13. Body composition (DXA) for AN patients and comparison group. (A) Total body fat mass baseline–3 years in AN (p<0.001), AN vs comparison group (p=0.026). (B) Trunk fat mass baseline–3 years (p<0.001), AN vs comparison group (p=0.044). (C) Lean mass index baseline–3 years in AN (p=0.191), AN vs comparison group (p=0.031).

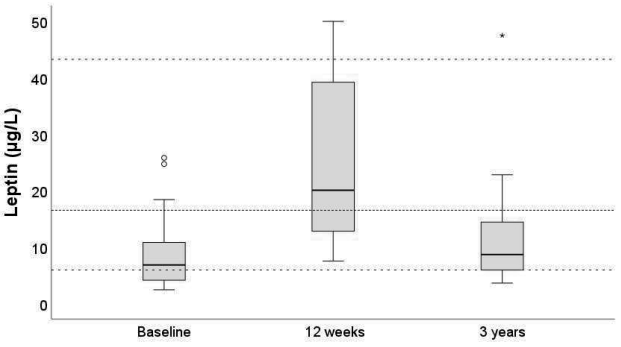


Figure 14. Leptin levels in AN patients over 3 years. Levels increased during nutrition treatment ($p<0.0001$) but was stable between baseline and 3 years ($p=0.19$). Dotted lines represent the 5th, 50th, and 95th percentile for 18-year-old healthy girls according to Lausten-Thomsen et al. [192].

After 3 years, BMI and body composition parameters were still not related to the *FTO* genotype. There was, however, a tendency towards more stable BMI in the TT group and a wider BMI range for the TA and AA groups, including three individuals with a pronounced increase in BMI after 3 years (figure 15). The *FTO* data from the 3-year follow-up has not been published previously.

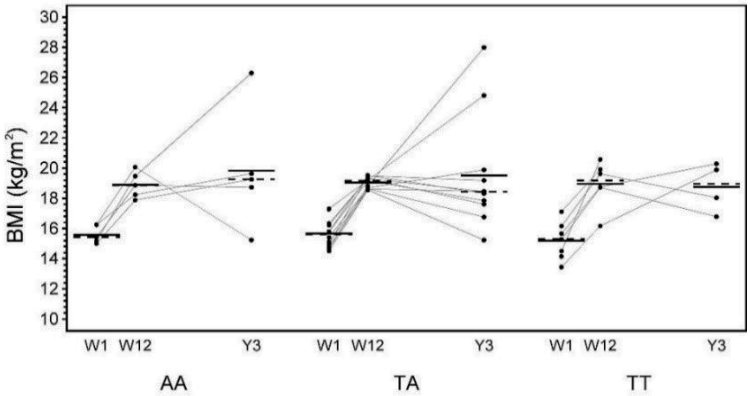


Figure 15. BMI for AN patients categorized by FTO genotype. The solid horizontal lines represent the mean BMI and the dashed lines (---) the median BMI for the FTO genotypes. W1 = week 1 (start of nutrition treatment), W12 = week 12 (end of nutrition treatment), and Y3 = 3-year follow-up.

6.1.3 BONE MASS IN ANOREXIA NERVOSA

After 3-years, BMC and aBMD for total body, lumbar spine, and total hip were stable, but all parameters were significantly lower for the AN cohort than for the comparison group. aBMD of spine, total hip, and left leg and BMC of total body, lumbar spine, and left leg were all lower in the AN cohort than the comparison group, even after adjustment for BMI. At follow-up, 15% of the AN subjects, but none of the healthy comparison individuals had low bone mass (aBMD Z-scores <-2SD), figure 16.

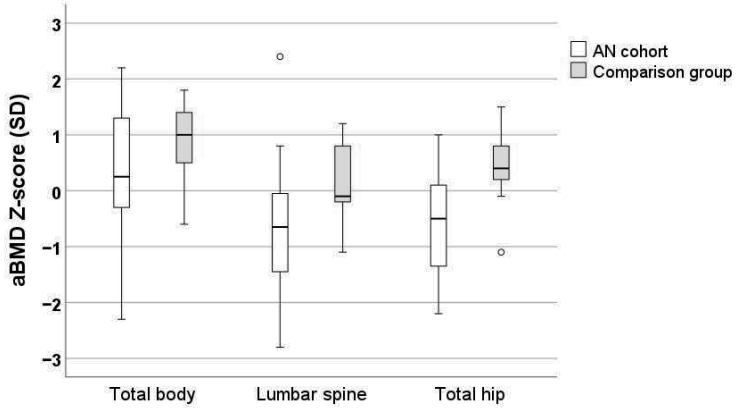


Figure 16. DXA aBMD Z-scores for AN subjects and comparison group at 3-year follow-up. aBMD of lumbar spine and total hip were significantly lower in AN individuals than the comparison group, p=0.017 (lumbar spine) and p=0.0005 (total hip). After adjustment for BMI, significant difference for total hip, p=0.007.

Results paper III

- From discharge after intensive nutrition treatment until follow-up after 3 years, BMI and body composition were stable.
- There were positive changes in bone turnover markers and sclerostin after 3 years in AN patients.
- Despite this, aBMD was low in the AN cohort compared to the healthy comparison group and trabecular vBMD decreased.

Two individuals were identified with low bone mass of lumbar spine at inclusion, but normalized aBMD at follow-up, coinciding with a normal BMI and regular menses. Three other AN individuals were identified with low bone mass of lumbar spine at 3-year follow-up, of which two women had a long duration of AN (diagnosis at age 11 and 12 years, respectively) and low BMI at follow-up (15.2 kg/m^2 and 16.8 kg/m^2). The third individual had low aBMD at follow up despite a shorter duration of AN as well as a normalized BMI and regular menses. Trabecular vBMD decreased over 3 years in the AN cohort and was lower than in the comparison group, although not after adjustment for BMI (figure 17).

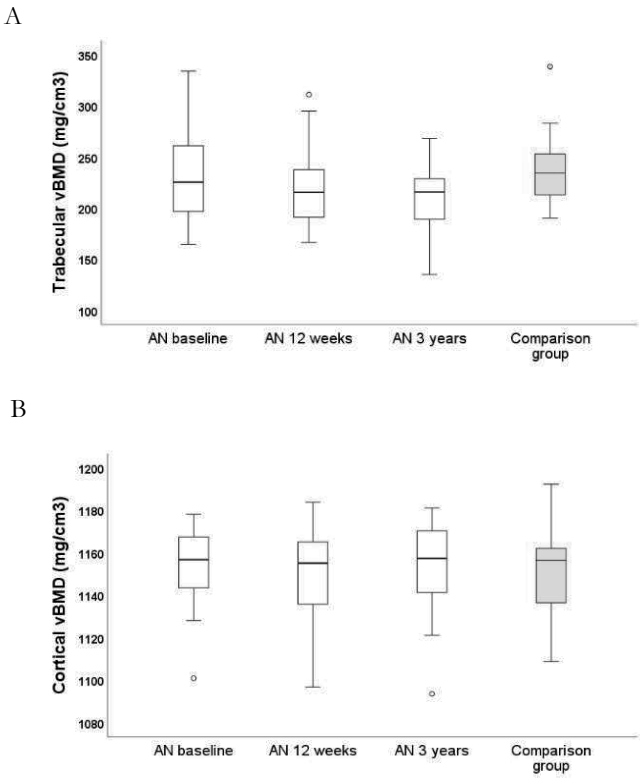


Figure 17. pQCT results for AN cohort and comparison group. (A) Trabecular vBMD decreased from baseline to 3 years in AN, p=0.003. AN vs comparison group, p=0.0042, after adjustment for BMI p=0.203. (B) Cortical vBMD was stable over 3 years; no difference between AN and comparison group.

Previous fractures were reported by 50% of the AN cohort and 35% of the comparison group, although the difference was not significant. The level of physical activity, evaluated by the IPAQ questionnaire [185], did not differ between the AN cohort and the comparison group at 3-year follow-up.

At 3-year follow-up, 40% of the women still had ongoing treatment for AN. Fourteen patients were amenorrheic (defined as absence of ≥3 consecutive menstrual cycles) at baseline and six of these still after 3 years. Nine women had regular menses and five were on hormonal contraceptives at 3-year follow-up. For the amenorrheic women, aBMD for total body, hip, spine, and left leg were lower than in controls. However, even in individuals with resumption of menses, both spine and total hip aBMD as well as trabecular vBMD were lower than in the comparison group.

The bone formation markers BALP and osteocalcin increased during nutrition treatment, while the resorption marker CTX was stable. From 12 weeks to 3 years, BALP was stable but osteocalcin decreased to a level that was not significantly different from baseline. The bone resorption marker CTX decreased from 12 weeks to a normalized level at 3 years. Sclerostin decreased during 12 weeks of nutrition treatment and had further declined at the 3-year follow-up (figure 3, paper III). Sclerostin was associated to BMI, figure 18.

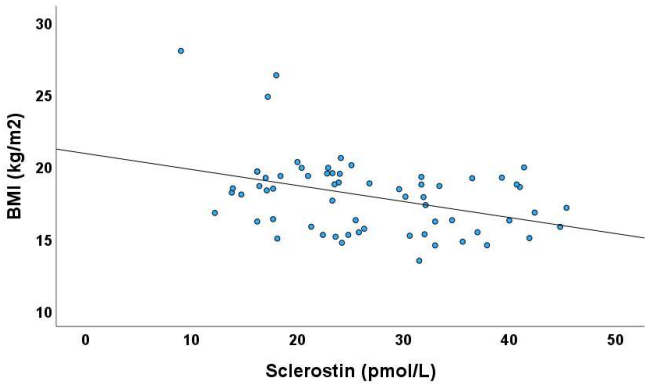


Figure 18. Sclerostin vs BMI in AN patients over 3 years, showing a negative correlation (r=-0.337, p=0.006).

DXL measurements were performed at 3-year follow-up; this data has not been published previously. Both calcaneal BMC and aBMD decreased in the AN cohort over 3 years and were significantly lower than in the comparison group, even after adjustment for BMI (figure 19).

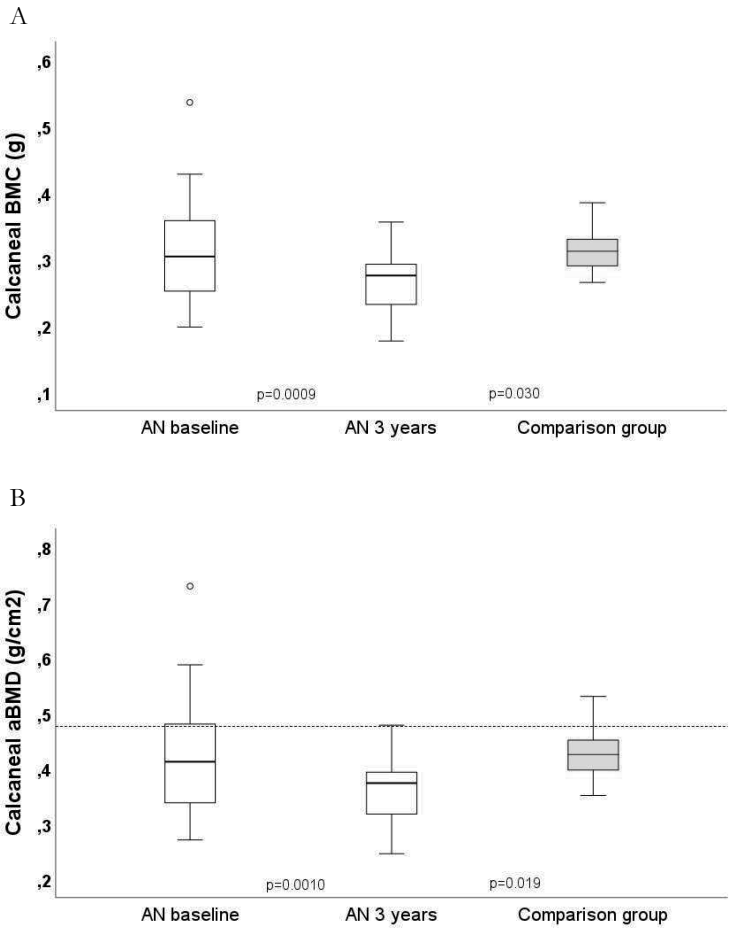
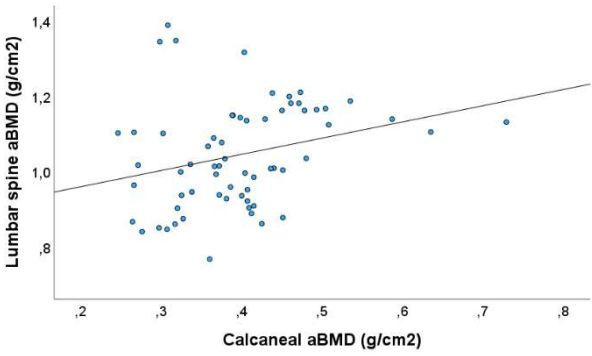


Figure 19. DXL measurements of calcaneus in the AN cohort and comparison group. (A) BMC decreased in AN over 3 years (p=0.0009) and was lower in AN than in the comparison group after 3 years (p=0.030, adjusted for BMI). (B) aBMD decreased in AN over 3 years (p=0.0010) and was lower in AN than in the comparison group (p=0.019, adjusted for BMI). The dotted line represents the reference mean aBMD of 0.48 g/cm² for women aged 20–29 years [193].

A



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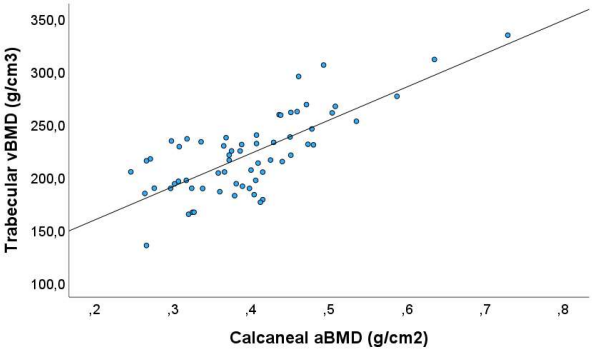

Figure 20. Correlations between bone mass of different sites in AN patients. (A) Lumbar spine aBMD vs calcalneal aBMD, showing a positive correlation (r=0.365, p=0.003). (B) Trabecular vBMD vs calcaneal aBMD, also showing a positive correlation (r=0.642, p<0.001).

6.2 THE MODIFIED ATKINS DIET (PAPER IV)

Thirty-eight children (21 girls, 17 boys, median age 4.4 years (range 0.4–17.2 years)) were included when they started MAD treatment for intractable epilepsy, GLUT1-DS, or PDHCD. Twenty-nine children continued MAD treatment for 24 months, including all children with GLUT1-DS and all except one child with PDHCD.

Thirty patients (79%) had seizures prior to study start. The reduction of seizures in these patients is described in Table 6. In four children, treatment was discontinued due to insufficient effect on seizures between 6 and 12 months. Five children discontinued treatment between 12 and 24 months; for two of these the reason was insufficient effect on seizures.

Table 6. Seizure reduction in children with seizures at baseline remaining on MAD at 6, 12, and 24 months.

	6 months (*n*=30)	**12 months** (*n*=26)	**24 months** (*n*=22)
Seizure free	8 (27%)	8 (31%)	8 (36%)
>90% improvement	3 (10%)	3 (12%)	4 (18%)
50–90%	6 (20%)	8 (31%)	4 (18%)
1–49%	8 (27%)	5 (19%)	5 (23%)
No improvement	5 (17%)	2 (8%)	1 (5%)

Median 3-hydroxybutyric acid increased from 0.05 mmol/L at baseline to 2.35 mmol/L (0.42; 5.20 mmol/L) after 6 months and was then stable over 24 months. pH was stable and within the normal range over the study period. Blood lipid levels (total serum cholesterol, low-density lipoprotein, high-density lipoprotein, and triglycerides) were all stable over 24 months. 25(OH)D levels were adequate and stable over the study period of 24 months. No child was vitamin D deficient, but two were vitamin D insufficient (25(OH)D <50 nmol/L) at baseline. Fourteen children (37%) were supplemented with vitamin D during the study period and after 24 months no child was vitamin D deficient, but one was vitamin D insufficient.

Median weight SDS was stable over the study period: -0.4 at baseline and 0.2 after 24 months. Median height was stable from baseline to 24 months: -0.4 SDS and -0.3 SDS, respectively (p=0.10). The range in BMI at baseline was wide: -3.3 to 4.5 SDS. BMI SDS increased from baseline to 24 months, from 0.2 to 0.7 (p=0.0045).

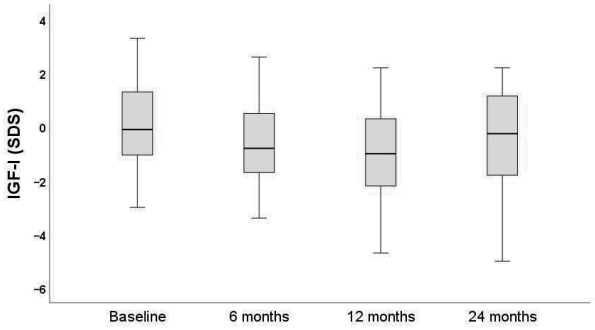

Figure 21. IGF-I in children on MAD over 24 months. There was a significant decrease from baseline to 6 months (p=0.003) and from 6 months to 12 months (p=0.02), followed by an increase from 12 to 24 months (p=0.009). From baseline to 24 months, IGF-I was stable, (p=0.16).

Four patients were treated with MAD during their puberty. Visual inspection of their growth charts by a pediatrician clarified that the growth spurt was not negatively affected in any of these patients. For one patient (girl, 9.5 years at baseline), the start of the pubertal growth spurt coincided with the start of MAD treatment.

Twenty-three patients (61%) were ambulatory at baseline. One patient (girl with PDHCD) was able to walk after 6 months of MAD treatment. DXA measurements were performed in 12 patients and DXL measurements were performed in 19 patients at baseline and after 24 months (Eight patients were measured with both DXA and DXL.) Ten (83%) of the children with DXA measurements and 12 (63%) of those with DXL measurements were ambulatory at baseline.

Bone health in this cohort was impaired to some extent, with aBMD Z-scores for total body and/or spine <-1 SD in three (25%) of the children at baseline. One child had an aBMD Z-score ≤-2 SD for total body and lumbar spine, both at baseline and after 24 months. For another child, aBMD decreased during the study period and the aBMD Z-score was ≤-2 SD for total body and lumbar spine after 24 months; this patient sustained a femur fracture during the treatment period. Both of these patients were non-ambulatory.

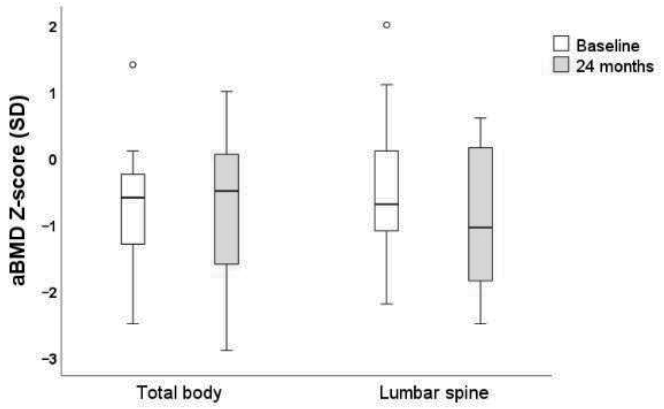

Figure 22. Bone mass (DXA) in children on MAD at baseline and after 24 months. Total body less head and lumbar spine aBMD Z-scores were stable over 24 months (p=0.25 and p=0.41, respectively).

Both TBLH, lumbar spine, and total hip aBMD were stable over 24 months. aBMD Z-scores for total body and lumbar spine are presented in figure 22. In addition, total fat mass and lean mass were stable over the study period. The DXL measurements showed that calcaneal BMC and aBMD increased over 24 months, but since calcaneal height also increased, BMAD (mg/cm^3) was stable. Most children had lower calcaneal BMAD than the lower limit of the reference interval [125].

The body composition parameters fat mass, lean mass, and lean mass index (kg/m^2) were stable over 24 months. According to reference values [16, 194], fat mass percentage in these children was comparable to the normal reference, both at baseline and after 24 months, whereas lean mass index was below the reference median in seven of nine children at baseline and in seven of eight children after 24 months.

Results paper IV

- MAD was as effective as KD in reducing seizures in children with intractable epilepsy, GLUT1-DS, and PDHCD.
- Weight and height SDS were unaffected during MAD treatment for 24 months, while BMI increased and body composition was stable.
- Bone mass measured by DXA was stable over 24 months, probably related to increased BMI and the lower level of acidosis.

7 DISCUSSION

The research in this thesis enhances our current understanding of the effects of two different nutrition treatments on bone health, body composition, and growth in children with epilepsy and young women with anorexia nervosa. Both nutrition treatments proved to be efficacious for their respective purpose: weight restoration in women with severe AN and seizure reduction in children with intractable epilepsy. We were able to show that treatment with MAD did not negatively affect longitudinal growth or bone mass in children during treatment with this diet for 24 months. For women with AN, BMI was stable at the group level at follow-up 3 years after nutrition treatment, and 60% no longer had ongoing treatment for AN. However, having had AN during adolescence still resulted in lower bone mass in these women compared to healthy women in the comparison group. These results strengthen the importance of the adolescent years for bone mass acquisition and suggest that recovery from AN, and normalization of the hormonal systems, is a time-consuming process.

Intensive nutrition treatment in anorexia nervosa

The results from paper I demonstrate that intensive weight gain treatment with a high-energy diet from start is effective in terms of weight recovery and at the same time a safe treatment. The mean weight gain over the first 6 weeks in our studies was 6.1 kg; from week 6 to week 12, the mean weight gain was 3.7 kg, in accordance with current guidelines for several European countries, where a minimum weight gain of 0.5 kg/week is recommended [195]. Recently updated guidelines from the American Psychiatric Association suggest even more intensive nutrition treatment, with a target weight gain of 1.0 to 2.0 kg/week for patients in inpatient programs, but with individually adapted treatments and target weights [144]. The median daily energy intake in the women with AN was 3351 kcal (75 kcal/kg) from start, which was higher than the initial caloric intake usually practiced in 2012–2014 when these studies were carried out; on the other hand, this is well in line with the more recent recommendations for nutrition treatment in AN [145, 195]. None of the AN patients in studies I and II presented symptoms of refeeding syndrome.

From 2015, there was an increase in the proportion of admissions for more severe or extreme AN at the Child and Adolescent Psychiatry department at the Queen Silvia Children's Hospital. In several of these more serious cases, the treatment protocol had to be adapted to a more individualized treatment regimen in which the patient was prescribed a lower energy intake during the first week, which was then increased in several steps. Since the treatment of these patients no longer

followed our study protocol with a high-energy diet from treatment start, it was decided that no further patients should be recruited to the study.

To enable complete or sustained recovery, both weight recovery and behavioral recovery are necessary. Our group has previously studied changes in food attitude in this cohort of AN patients and found that their anxiety related to food gradually decreased during intensive nutrition treatment, despite the large weight gain [184].

After 3 years, 11 patients (55%) were considered weight restored (BMI >18.5 kg/m^2). However, 80% of the women had a daily energy intake that was below their estimated needs (2250 kcal/day), and 85% had suboptimal intake of vitamin D, iron, and folate [196]. After discharge from the hospital, 18 women (90%) continued specialized outpatient treatment; at follow-up after 3 years, 40% of the patients were still in active specialized eating disorder treatment and 35% met the criteria for AN diagnosis. That a significant proportion of the patients still had an eating disorder diagnosis after 3 years is consistent with previous findings that recovery from AN is a slow process. A follow-up study of Swedish adolescents with AN found that after 18 years, 12% met the criteria for an eating disorder (half of which met the criteria for AN) [197]. The recently published 30-year follow-up of the same cohort demonstrated that 19% had a current eating disorder (AN diagnosis in 6%). Relapses in AN occurred during follow-up and 17% fulfilled the criteria for AN at some point between these studies. After 30 years, 64% were considered fully recovered from eating disorder symptoms [136].

The modified Atkins diet

For many patients, the less restrictive MAD treatment is a more appealing treatment option than the classic KD, and the results from paper IV support previous studies showing that the efficacy of MAD in seizure reduction is comparable to results presented for the classic KD [198, 199]. The proportion of patients with ≥50% seizure reduction (responders) was 53% after 24 months, and only six (20%) patients discontinued the treatment due to inadequate effect on seizure frequency. These results are also in line with other studies on MAD treatment [177-180, 200]. The effect in patients with GLUT1-DS was especially satisfying: all patients with this condition and with seizures at baseline were free from seizures after 6 months and continued to be seizure-free throughout the study period of 24 months, also consistent with previous studies [201, 202]. A recently published meta-analysis, however, demonstrated a higher percentage of responders to classic KD than to MAD, although the percentage of patients with seizure frequency reduction >90% and seizure freedom was comparable between the diets [182]. With the aim of increasing retention and palatability, new diets have since been developed, mimicking the effects of the classic KD. Currently,

there are four major KDs: the classic KD, MAD, the low glycemic index diet, and the medium-chain triglyceride KD. Publications from the USA in recent years place MAD and the low glycemic index diet under an umbrella term of "modified ketogenic diets" [203]. According to a review by Wells et al. [176], there is not a clearly superior diet in terms of seizure reduction in children. A considerable proportion of patients discontinue treatment due to lack of efficacy, while responders often stay on the diet for a prolonged time. In an RCT by Sondhi et al. [181], the proportion of responders was similar between classic KD, MAD, and the low glycemic index diet.

7.1 BODY COMPOSITION AND GROWTH

Body composition and the *FTO* gene in anorexia nervosa

The aim of nutrition treatment in AN is weight recovery, and **papers I and II** demonstrate that body weight and BMI increased significantly during the nutrition treatment period (median 9.8 kg and 3.4 kg/m^2 respectively). Total body fat mass increased and the range in total fat mass after 3 years was wide in AN patients, from 8.7% to 40.6%. Trunk fat mass accounted for a large part of the increase in fat mass, which is consistent with the results of Mayer et al. [204], who found an abnormal distribution of body fat in women with AN after 10 weeks of treatment, with trunk fat mass greater in weight-recovered AN patients than in controls. In a review by El Ghoch et al. [205] the same pattern was described, with a normalization of the fat distribution after long-term maintenance of weight restoration. Grinspoon et al. [206] found a relation between trunk fat accumulation and cortisol concentrations during weight recovery in AN patients. A meta-analysis by Hübel et al. [207] showed that total body fat was approximately 50% lower in AN patients than controls, and reflected by the leptin concentrations. The same study also described that lean mass was lower in AN patients than controls, even after treatment, and in weight-recovered AN patients, which is consistent with the results from **paper III**.

In **paper III**, leptin levels were low at baseline, since the secretion of leptin is reduced in parallel with the reductions in fat mass [208]. Leptin increased with refeeding and weight recovery, strongly correlated to the increase in fat mass, which is also in line with previous knowledge [146, 209]. After 3 years, leptin decreased to a level corresponding to the baseline value. Treatment with recombinant leptin has been tested in women with hypothalamic amenorrhea due to low weight or strenuous exercise and found to be associated with improved

reproductive function, but also a decrease in fat mass, which makes recombinant leptin a less appealing treatment option for women with AN [210].

The high-energy diet for AN was effective in all patients, and we could not demonstrate any difference in BMI or body composition parameters between the *FTO* genotype groups in **paper II**. As described previously, variants within or near the *FTO* locus might be associated with a greater food intake, a preference for energy-dense food, less sensitivity to satiety, and a propensity for loss-of-control eating [32-35, 211]. Since all patients were offered the same menu during the treatment period, the effect of the *FTO* genotypes was probably removed in **paper II**. But after 3 years with a self-controlled food intake, we could still not demonstrate any differences in weight, BMI, or fat mass between the *FTO* genotype groups. After 3 years, a trend to more stable BMI in the TT genotype group was noticed, though it was not significant. A few studies have focused on the correlation between the *FTO* genotype and eating disorders, but the results diverge. One study described an association between the *FTO* rs9939609 A allele and AN and bulimia nervosa [40], another study found that in individuals with eating disorders, the presence of the A allele represents a risk factor for developing emotional eating and binge eating [41]. In contrast, Palmeira et al. [43] tested the possible association of *FTO* rs9939609 with binge eating disorder and found a lower frequency of the A allele in these patients than in controls.

The modified Atkins diet, growth, and body composition

In **paper IV**, we demonstrated that in children and adolescents on MAD treatment, weight and height increased significantly between every visit, proving that these children continued to grow. Accordingly, weight and height SDS were stable over 24 months. There was a wide range in BMI at baseline, and this study includes some participants with underweight and some with obesity. BMI for the cohort increased during the study period, but after 24 months the range in BMI was smaller, from -0.9 to 2.6 SD. The interpretation of this might be that the MAD treatment was beneficial for all patients, and that both underweight and obesity were well managed during the treatment period. El Rashidy et al. [183] found stable BMI in children on MAD treatment for 6 months, but after 6 months, BMI was significantly lower in this group than in patients on the classic KD. Also, Kossoff et al. [177] presented stable BMI in children on MAD treatment for 6 months. For comparison, one review found that the impact on growth in children on the classic KD was still not established, with some studies showing a positive effect, while negative effects were observed after long-term KD treatment (>12 months) [212]. Two different studies on the classic KD demonstrated a decline in height Z-scores after 2 years [174, 213], with one of

these studies showing a catch-up and return to the expected weight and height percentile 1 year after diet discontinuation [213].

As far as we know, **paper IV** is the first study to evaluate long-term growth in children on MAD treatment. Although protein intake is not restricted with MAD, an intake above the child's or adolescent's needs may impact the ability to maintain ketosis, and thus the protein content of the diet was calculated based on needs related to age and body weight. IGF-I decreased during the first year of treatment, coinciding with the great metabolic changes occurring when starting MAD treatment, probably related to the ketotic state but also to the altered protein content of the food, and correlated to the height SDS for the same period. However, from 12 to 24 months there was an increase in IGF-I, probably reflecting a metabolic stabilization after prolonged treatment, coinciding with the increase in BMI SDS from 12 to 24 months. Accordingly, linear growth was not affected over 24 months.

7.2 BONE HEALTH

Bone health in anorexia nervosa

In **paper III**, the median age of the AN cohort was 24.0 years (range 19.2–27.8 years), meaning that these women had passed the period of peak bone accrual but that PBM was likely not yet achieved, especially for the spine, according to the findings of Berger et al. [69]. In the AN cohort, aBMD and BMC of total body, lumbar spine, and total hip were all stable over 3 years, during a period when one would expect a slight increase in BMC and aBMD values in healthy individuals, especially for the spine, according to the evidence above. The differences in bone parameters between our AN cohort and the comparison group might thus be explained by greater gains in bone mineral in healthy young women than in girls with AN, even among weight-recovered subjects, which has been described previously [214].

In **paper III**, trabecular vBMD (measured by pQCT) decreased while cortical bone was stable in the AN cohort, which could to some extent be related to the higher bone turnover of trabecular bone than cortical bone as a result of metabolic and hormonal fluctuations [59]. Trabecular vBMD was significantly lower in the AN cohort than in the comparison group, although not significant after adjusting for BMI. Cortical vBMD, however, was stable and did not differ between the AN cohort and the comparison group. According to previous studies, trabecular bone accrual peaks during mid puberty, while cortical bone

accrual peaks later and continues into the third decade of life, which could be relevant for the interpretation of these results [74, 75]. Lower trabecular vBMD but higher cortical vBMD using pQCT in young women with AN, compared to healthy controls, has been reported previously [215]. One study pointed at compromised trabecular vBMD of distal tibia in women after stable recovery from AN for 27 years, compared to controls [216]. Trabecular bone score, a method evaluating the image textures off lumbar spine DXA without exposing the individual for additional radiation, is a measure of bone quality that can be used to predict fractures in adults. In a recently published paper, trabecular bone score increased in adolescent girls with AN during two years of treatment but was still compromised compared to healthy controls [217].

Singhal et al. [218] reported lower cortical vBMD, measured by HRpQCT, in AN patients with lower BMI and longer duration of AN. The same study found that periosteal and endosteal circumference in AN did not differ from controls, but that the cortical thickness was lower in AN. In **paper III**, both the periosteal and endosteal circumference increased over 3 years, while the cortical thickness decreased, but none of these parameters differed from the comparison group. Many differences in bone parameters between the AN cohort and the comparison group were no longer significant after adjusting for BMI, which underscores the major impact of low weight on these parameters.

Callréus et al. [219], presented normative DXA data for 25-year-old Swedish women, where aBMD was generally higher compared to NHANES and other reference cohorts. According to this reference, aBMD for lumbar spine and total hip in the comparison group in paper III matched the reference mean well, whereas aBMD Z-scores for those sites in the AN cohort were lower than calculated based on the NHANES reference mean, approximately -1 SD for both lumbar spine and total hip compared to -0.7 SD (lumbar spine) and -0.5 SD (total hip).

In line with decreased trabecular vBMD and the low Z-scores for lumbar spine in **paper III**, aBMD and BMC of the calcaneus (a bone constituted of predominantly trabecular bone), decreased from baseline to 3 years and were lower in the AN cohort than in the comparison group. Studies using DXL measurements of AN patients are lacking, but these results are in line with a recently published paper in which quantitative ultrasound was used for evaluating bone mass at the heel, which was found to be correlated to aBMD at all bone sites and significantly decreased in AN patients (mean 22.8 years) compared to controls [220].

The fracture incidence in childhood and adolescence did not differ between the AN cohort and the comparison group in **paper III**. In contrast, Faje et al. [221] demonstrated that the prevalence of fractures was higher in girls with AN (age 12–22 years) compared to healthy controls, independent of whether their aBMD was lower. The same pattern was found in a similar study of adult women with AN [222], and it can probably be explained by bone strength being associated with other parameters not assessed by DXA, such as microarchitectural parameters [218]. A recently published paper, though, found that women with AN and previous fractures had greater bone marrow adipose tissue compared to women with AN without fractures, while aBMD and bone microarchitecture parameters did not differ between the groups [223]. The lifetime risk of fractures in women with AN is increased up to threefold, with greatest risk of fractures of the distal forearm, spine, and hip [222]. The Endocrine Society [224] recommends aBMD measurements by DXA in any adolescent girl or woman with ≥6 months of amenorrhea, earlier in those with a history of severe malnutrition or skeletal fragility. Since the course of AN can fluctuate, with periods of recovery and relapse, serial measurements may have greater value for the prediction of fracture risk than a single measurement. In our 3-year follow-up, patients were informed about their DXA results and, if bone mass was impaired, the individual was referred to the osteoporosis department at Sahlgrenska University Hospital for further follow-up.

In **paper III**, bone mass was compromised in women with AN, both those with resumption of menses and those who were still amenorrheic, compared to the healthy comparison group. There was no catch-up in bone mineral accretion with recovery over 3 years. Trabecular vBMD was stable in patients with regular menses at 3-year follow-up but decreased significantly over 3 years in patients who were amenorrheic at follow-up. Grinspoon et al. [159] reported that the duration of amenorrhea and age at menarche are predictive factors for spine aBMD, indicating a greater effect of estrogen deficiency at this site than other sites. Miller et al. [225] found that total body and lumbar spine aBMD were significantly lower in amenorrheic than menstruating women with AN. Nevertheless, the association of amenorrhea and bone health in AN has been debated and results are inconsistent. One study demonstrated that weight restoration increased aBMD independently of resumption of menses in AN [226] and another that bone density was compromised to the same extent in amenorrheic and menstruating AN women [227]. Legroux-Gérot et al. [228] found that the duration of amenorrhea is a major factor for the variance in aBMD, but that leptin is also a predictive factor of spinal aBMD in AN patients. In women with AN, hypoleptinemia has been associated with low aBMD of spine and hip independently of BMI [157]. In **paper III**, leptin levels were reduced both

at baseline and after 3 years compared to the reference for healthy 18-year-old girls [192].

The results from **paper III** thus strengthens the claim that an improved nutritional status alone is not enough to normalize bone health in AN. Several hormonal treatments for AN have been discussed and evaluated. Oral contraceptive pills (containing estrogen and progesterone) do not improve aBMD in AN [229, 230], but Misra et al. [231] demonstrated that physiological transdermal estrogen replacement was effective in increasing, but not normalizing, spine and hip aBMD Z-scores in adolescent girls with AN. The mechanism behind this difference might be that oral estrogen is metabolized in the liver and may inhibit IGF-I production and increase IGF-BP3, thus limiting free IGF-I [232].

The modified Atkins diet and bone health

The results from **paper IV** contribute new insights about bone health in children on MAD. The bone health in this cohort turned out to be impaired to some extent, with aBMD for total body and/or lumbar spine below the mean reference value for a majority of the subjects at baseline. One patient had low bone mass both at baseline and after 24 months, and for another patient aBMD went from normal to low during the study period. Both these patients were non-ambulatory and, hence, the lower aBMD Z-scores are probably mainly related to low mechanical loading and low muscle activity. In contrast, the results from **paper IV** are positive, revealing that aBMD Z-scores of total body and spine, as well as BMC for the same localizations, were stable in this cohort during the treatment period. This may be explained by the more liberal protein intake and higher total energy intake with MAD than with the classic KD and the stable body composition and adequate vitamin D levels demonstrated during treatment. Also. 3-hydroxybutyric acid levels were lower than with the classic KD, and pH was normal and stable over the study period, favorable for bone acquisition. The results from paper IV thus contradict previous results, where a progressive loss of BMC and BMD Z-scores in children treated with classic KD were reported [175, 233]. The absolute values for BMC and aBMD of total body, lumbar spine, and total hip were stable over 24 months. Reference curves for children aged 1–17 years by Kalkwarf et al. [234, 235] indicate that both BMC and aBMD increase with age during childhood and adolescence, and that the variability increases with age. Accordingly, since the children in **paper IV** who underwent DXA had not yet reached their PBM, one would expect an increase in BMC and aBMD as a reflection of bone accrual.

The evaluation of bone health in children on MAD in **paper IV** is strengthened by the DXL measurements, which could be performed in a larger proportion of the patients. The DXL technique has several advantages, and for children <5 years (where DXA often cannot be performed, and reference data is lacking) DXL is a highly suitable method with reference data available for children [125]. Although calcaneal BMC and aBMD were low in all patients, there were significant increases in both parameters over 24 months, while BMAD was stable, consistent with bone accrual during growth. Calcaneal aBMD showed a positive correlation with both lumbar spine aBMD and trabecular vBMD. Another study in children and adolescents (2.2 to 20.6 years) concluded that calcaneal aBMD values by DXL correlate well with DXA values, and that the two techniques can effectively identify the same individuals with low aBMD [236]. If we had succeeded in performing DXA scans in all patients, we may have been able to detect an increase in BMC and aBMD of total body, lumbar spine, and total hip related to the expected bone acquisition for their age. Among the 23 children examined with DXA and/or DXL, 16 were ambulatory and seven were not. Due to the low numbers, statistical conclusions could unfortunately not be made for the differences in bone mass parameters between ambulatory and non-ambulatory children.

Vitamin D and PTH

In both children on MAD and women with AN on intensive nutrition treatment, 25(OH)D levels were adequate during the treatment period and very few patients were vitamin D deficient. Supplemental intake of vitamin D and calcium was high among AN patients before nutrition treatment, which has been demonstrated previously [237]. In our studies, 37% of the children on MAD and 96% of the women with AN received vitamin D supplements during nutrition treatment. Hypercalcemia has been reported previously with the classic KD, related to the chronic ketotic state altering bone and calcium homeostasis, possibly by increasing bone resorption [238], but hypercalcemia was not seen in any child or adolescent in **paper IV**.

Papers I and III contribute to the body of knowledge about vitamin D in AN patients in northern latitudes and strengthen the importance of vitamin D supplementation during nutrition treatment. The drop in 25(OH)D, although it was still to an adequate level, might be explained by low exposure to sunlight and the significant increase in fat mass during nutrition treatment. Decreased levels of 25(OH)D is seen in in obesity and has been related to storage of this fat soluble vitamin in adipose tissue [239] and also to volumetric dilution in a greater tissue volume in obese individuals, since 25(OH)D is distributed in serum, fat, muscle, and liver [240]. However, DiVasta et al. [241], found that the bioavailability of

vitamin D after a single dose of vitamin D_2 was comparable in women with AN and normal-weight controls. Decreasing 25(OH)D levels in AN patients during nutrition treatment has been described previously and has been presumed to be related to the above-mentioned factors [166] and to the reduced sunlight exposure during hospitalization [242].

The daily intake of vitamin D in paper I (1000–1400 IU) was well in line with the Nordic Nutrition Recommendations but did not meet the intake recommended by the Endocrine Society to maintain 25(OH)D levels >75 nmol/L in adults [103]. Recommendations for vitamin D supplementation during in-hospital nutritional treatment for AN are lacking. Misra et al. [243] recommended a daily intake of 800 IU in all AN patients, but the results from **paper I** thus favor a higher intake of vitamin D in AN patients during in-hospital nutrition treatment in the northern latitudes. Future studies are, however, needed to evaluate which dose should be recommended.

After 3 years, 25(OH)D was stable, but unfortunately, information about substitution during the follow-up period is lacking. Gatti et al. [244] observed a trend of higher hip aBMD values in AN patients with a 25(OH)D ≥75 nmol/L than in patients with 25(OH)D between 50 and 75 nmol/L. However, they concluded that adequate 25(OH)D levels alone are not enough to improve aBMD. As opposed to total 25(OH)D, free 25(OH)D was stable during nutrition treatment and then decreased, although within the normal range for the whole study period. Studies on free 25(OH)D in AN are limited, but in a small Swedish study there was no difference in 25(OH)D or free 25(OH)D between AN patients and healthy controls [245]. Measurement of estradiol would probably have contributed to our understanding about free 25(OH)D in **paper III**, since estrogen increases DBP synthesis [246]. One can speculate that the lower levels of free 25(OH)D in the AN cohort after 3 years could be related to hormonal changes and probably higher estradiol levels, since several patients had regained their menstrual function at the 3-year follow-up.

In **paper III**, PTH was measured and found to be stable and within the normal range, both during nutrition treatment and after 3 years. The biologically active form, n-oxPTH, was stable during nutrition treatment but showed an increase after 3 years. A review by Ursem et al. [247] speculated that n-oxPTH could possibly be used to distinguish whether elevated PTH is related to increased oxidative stress or parathyroid gland hyperactivity. Further, in conditions with high oxidative stress, such as chronic renal failure and chronic inflammation, n-oxPTH could be a clinically relevant parameter. Possibly, the increased n-oxPTH in the AN cohort at follow-up reflects decreased oxidative stress, but the role of oxidative stress in AN needs to be clarified in future studies.

Bone biomarkers in anorexia nervosa

Change in aBMD is a slow process, and measurement of bone biomarkers therefore contribute with a more immediate view of bone mineral status and deepen our understanding of the effects of weight recovery and hormonal alterations on bone. Previous studies have reported that adolescents with AN have lower levels of bone formation markers and similar levels of bone resorption markers (indicating decreased remodeling) compared to normal weight adolescents [156, 248, 249]. Adults with AN have similarly suppressed levels of bone formation markers but, unlike adolescents, they have increased levels of bone resorption compared to healthy women [151, 250]. In **paper III**, the bone formation markers osteocalcin and BALP increased by 85% and 100%, respectively, after 12 weeks of nutrition treatment, consistent with previous results [251-253]. Oświęcimska et al. [254], in contrast, found that, while BALP increased after nutrition treatment, osteocalcin levels did not change. In **paper III**, the bone resorption marker CTX was stable during intensive nutrition treatment, while another study demonstrated a decrease in CTX during 3 months of refeeding in adult women with AN [252]. Altogether, these changes might exert a positive effect on bone and could be related to the increase in BMC that was demonstrated in **paper I**. After 3 years, CTX decreased to a level seen in healthy young women [255]. Osteocalcin decreased from 12 weeks to 3 years, to a stable level compared to baseline, and BALP was further increased at 3-year follow-up. Thus, even after 3 years there were changes in biomarkers that were positive for bone mass. But despite this and the normalized BMI at 3-year follow-up, BMC and aBMD did not increase.

Sclerostin in anorexia nervosa

In **paper III**, sclerostin decreased during nutrition treatment and further decreased after 3 years, resulting in a decreased inhibitory effect on Wnt signaling. This, in turn, leads to increased bone formation and decreased bone resorption and thus, improved conditions for bone acquisition [94]. To our knowledge, paper III is the first study prospectively evaluating sclerostin levels in AN during nutrition treatment and weight recovery. Previous studies have shown that sclerostin is negatively regulated by estradiol in adults, with higher levels of sclerostin in postmenopausal than premenopausal women [256]. In a study by Faje et al. [162], levels of sclerostin correlated with markers of bone formation and resorption in healthy girls, but this association was not seen in adolescent girls with AN. In the same study, an association between estradiol and sclerostin could not be verified in AN patients or controls. Increased sclerostin levels in AN compared to healthy controls have been described earlier, and found to be negatively correlated with BMI, fat mass, and lean mass [257]. In **paper III**, there were negative associations between sclerostin and BMI and fat mass in AN

patients, but no significant association with lean mass. In contrast, Amrein et al. [258] presented a positive association between sclerostin and BMI and fat mass in healthy adults. In **paper III**, sclerostin was not associated with cortical or trabecular parameters (pQCT) in AN. One previous study in healthy girls and boys aged 6–21 years, demonstrated an association between sclerostin and cortical porosity, but not trabecular bone.

7.3 STRENGTHS AND LIMITATIONS

Strengths

The major strengths of these studies are the prospective design and the strong clinical significance, with the aim of evaluating established nutrition treatments and their possible negative effects on children and adolescents. For papers I and II, the treatment period of 12 weeks was well controlled, and the patients were all on the same protocol. The intake of total energy and vitamin D was well documented, and all meals were supervised for 12 weeks. Paper II is the first study evaluating the impact of the *FTO* genotype in patients undergoing intensive nutrition treatment with the aim of gaining weight. The use of three different methods to evaluate bone health and body composition is another strength. For the DXA measurements, we made use of the existing robust reference data from healthy children. The extended laboratory assessments in paper III, with biomarkers of bone and mineral metabolism, constitute another strength. In addition, the small number of dropouts is a strength worth highlighting. In the 3-year follow-up, all patients were informed about their DXA results and, if bone mass was low, the patient was referred to the osteoporosis unit for follow-up.

For paper IV, the main strengths are the follow-up time of 24 months and that the patients were closely monitored at the same center.

Limitations

Clearly, there are some limitations with the studies included in this thesis. First, the statistical power and, thereby, the generalizability is limited due to the small number of included patients. The intensive nutrition treatment for severe AN is very staff intensive and, due to a limited number of beds at the eating disorder unit, only 25 patients were eligible for this treatment over a 2-year period. Since the treatment protocol was then changed to a more personalized treatment regime, with a shortened hospital stay for some individuals, inclusion of more patients was not possible in this study design. A control group from start would

have been favorable; however, it would have been highly unethical to prescribe healthy young women this high-energy diet aimed at gaining about 10 kg in body weight. For paper I, a limitation is that the vitamin D supplementation varied between the patients both before and during the treatment period. Free 25(OH)D was not analyzed in this study but was complemented by paper III, which included it. For paper III, one limitation is the incomplete information on nutritional status, interventions, and physical activity during the follow-up period. For resource reasons, we did not have the opportunity to follow the patients during this period. Thus, intervening therapies could be a potential confounder. Measurement of hormones would have enhanced the quality of paper III but were deprioritized due to practical circumstances, such as need for testing in the morning hours for estradiol and cortisol. Also, since these parameters were not included in the design of paper I, the value of such analyses after 3 years was considered limited. The cohort of paper IV is heterogeneous, with a variety of etiologies and a wide range in age and stage of development, making the interpretation complex. Another shortcoming with paper IV is the lack of a control group. It was, however, considered unethical to refrain from treating children with intractable epilepsy with MAD by including them in a control group. One significant limitation is that DXA results are lacking for a significant proportion of patients in paper IV. This is explained by the low age of some patients, non-assessable measurements due to artefacts from movement (sometimes spasticity), and in some cases, hospitalization, and no possibility to conduct the DXA investigation at the right time. Nonetheless, the DXL measurements can to some extent, compensate for the lack of DXA data.

8 CONCLUSIONS

PAPER I

The AN patients' 25(OH)D levels were adequate both before and after 12 weeks of nutrition treatment but decreased despite a daily vitamin D intake exceeding the recommendations; this can be explained by low exposure to sunlight and increased sequestering of vitamin D in fat mass. There were no correlations between vitamin D status and BMI, fat mass, or bone mass parameters. This study indicates the importance of a high intake of vitamin D during in-hospital nutrition treatment for AN, to maintain an adequate vitamin D status.

PAPER II

The nutrition treatment with high energy from start was effective in all patients, and there was no difference in weight gain, BMI, or fat mass between the *FTO* genotype groups (AA, TA, and TT). Hence, the hypothesis that the *FTO* genotype would be associated with the increase in body weight and fat mass could not be verified. According to these findings, the *FTO* genotype cannot be used to predict the response to nutrition treatment or to provide a basis for individualized nutrition treatment in AN.

PAPER III

The 3-year follow-up of AN patients demonstrated stable BMI and body composition, changes in markers of bone turnover, usually contributing to increased bone mass, and reduced sclerostin levels. Despite this, the trabecular bone density decreased, and most bone mass parameters were lower in the AN cohort than in the healthy comparison group. This study highlights the importance of early evaluation of bone health in young women with AN, with a structured long-term follow-up, even in those who have recovered.

PAPER IV

MAD was effective in reducing seizures. In contrast to our hypothesis, there were no negative effects on longitudinal growth or bone mass in children after MAD treatment for 24 months. This can be explained by increased BMI, stable body composition during the treatment period, and a lower 3-hydroxybutyric acid level. Reduced seizure frequency may lead to increased physical activity and consequently increased mechanical loading, which is also favorable for bone acquisition. Hence, MAD can be considered an efficient and safe treatment option in children and adolescents regarding growth and bone health.

9 FUTURE PERSPECTIVES

The findings from these studies need to be corroborated by future studies. Studies that would deepen current understanding of bone health in AN could include:

- Assessment of PBM in young adults with a history of AN during adolescence through long-term follow-up and repeated measurements in a large number of patients from different centers. This study design would also enable inclusion of male patients with AN, which is warranted since low bone mass is also a concern in male patients but rarely studied.

- Correlations between PBM and fractures during adolescence and later in life in individuals with AN, possibly by using the Swedish fracture registry.

- Measurements of bone microarchitecture using high resolution pQCT at the time of PBM and long-term when bone mass starts to decline, in relation to fracture incidence. This would give further insights about bone health throughout life in women and men with AN. An alternative method for evaluating skeletal quality could be trabecular bone score, a method evaluating the image textures off lumbar spine DXA without exposing the individual to additional radiation; this method has not yet been specifically used to assess bone health prospectively in AN.

- Future research could also focus on the endocrine dysregulation in AN and the individual predisposition for hormonal disturbances, with the aim of identifying individuals most at risk for somatic consequences of AN.

- The role of genetics, including the *FTO* gene, for the onset of AN and its significance for the course of AN needs to be further explored through GWAS.

Since AN during adolescence may be associated with somatic consequences throughout life, increased awareness and efforts in the prevention of eating disorders are essential, as well as early detection and treatment. The collaboration between child and adolescent psychiatrists and pediatricians could be improved,

preferably by introducing multidisciplinary team discussions about seriously ill AN patients. National Swedish guidelines for vitamin D and calcium substitution in AN and for follow-up with DXA measurements need to be developed.

Future studies of growth and bone health in children and adolescents treated with MAD could include:

- Long-term evaluation of bone health, evaluating PBM in young people treated during childhood and/or adolescence. Out of the 29 children in this thesis who were treated with MAD for 24 months, 21 are still on treatment with the MAD or modified ketogenic diet. A follow-up of growth and bone mass in these patients in the near future, after a median of 10 years of treatment, would be very valuable.

- Multicenter studies including more patients would enable evaluation of additional parameters affecting bone health in this cohort, such as walking ability and physical activity. This study design would also allow the inclusion of more patients with the same diagnosis, bridging the diminished generalizability related to various etiologies among these patients.

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