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Effects of growth on childhood bone status:

A study in the Generation XXI birth cohort

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Ao abrigo do Art.º 4º, § 2º do Decreto-Lei n.º 707/2018, fazem parte desta dissertação as seguintes publicações:

- I. Gestational Weight Gain and Offspring Bone Mass: Different Associations in Healthy Weight Versus Overweight Women
- II. Early Childhood as a Sensitive Period for the Effect of Growth on Childhood Bone Mass: Evidence from the Generation XXI Birth Cohort
- III. Weight Trajectories from Birth and Bone Mineralization at 7 Years of Age
- IV. Bone Formation and Resorption Markers at 7 Years of Age: Relations with Growth and Bone Mineralization
- V. When to Stop Looking at a Cause: an Application to Adiposity and Bone Physical Properties in Children

Ao longo da elaboração da presente dissertação, colaborei ativamente na definição e operacionalização das hipóteses em estudo e dos objetivos a responder em cada um dos artigos, bem como na análise estatística dos dados e interpretação dos resultados. Participei na recolha dos dados durante a reavaliação aos 7 anos de idade da Geração XXI. Fui responsável pela redação da versão inicial de todos os manuscritos e participei ativamente na preparação das suas versões finais.

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Abstract

Background

Fragility fractures constitute a major public health problem associated with considerable and growing individual, societal and economic burden. One of the main modifiable factors that determine fracture risk is bone strength that depends on bone size, morphology and material properties. As with many other chronic diseases, the approach currently used to explain the etiology of suboptimal bone strength is built on the premise that there is important tracking of this characteristic throughout the life course, modulated by gene-environment interactions. Body size is recognized as one of the major determinants of bone fragility and strong cross-sectional relations between body size and composition and bone properties have been clearly established since pediatric ages. However, important issues regarding longitudinal effects of early life growth on childhood mineralization remain to be clarified. Namely, there is still no clear understanding of the impact of maternal gestational weight gain (GWG) on offspring bone physical properties. Additionally, it is unknown whether knowledge of children's growth trajectories since birth improves the statistical prediction of bone mineralization or turnover during the first decade of life. Finally, the potential for adiposity to specifically influence bone physical properties in children remains to be clarified.

Objectives

In the present work our objectives were:

1. To quantify the associations between gestational weight gain and bone mineralization in offspring, testing early pregnancy body mass index as an effect modifier (Paper I)
2. To characterize the impact of early life growth on bone mineralization in childhood by:
 - a. Identifying sensitive periods for the effect of early growth on childhood bone mass (Paper II)
 - b. Assessing whether different trajectories of weight gain since birth influence childhood bone mineralization (Paper III)
3. To describe markers of bone turnover in generally healthy children and assess their correlations with anthropometric growth and bone mineralization (Paper IV)

4. To test the potential for adiposity to specifically influence bone mass in children, using common causes as a benchmark (Paper V)

Methods

We used data from the Generation XXI, the first Portuguese birth cohort study assembled in Porto. All women who delivered live-born children with gestational age above 23 weeks in one of the five public units in the metropolitan area of Porto between April 2005 and August 2006, and whose residence was in the units' catchment area were eligible for recruitment. Seventy percent of eligible mothers were invited and, of these, 91.4% accepted to participate. All 8647 children enrolled at birth were invited to attend follow-up assessments at ages four (2009-2011) and seven (2012-2014) years (86.3% and 79.7% participation, respectively).

At baseline, in the first 24 to 72 hours after delivery, trained interviewers were responsible for presenting the study and inviting mothers. Subsequent follow-up evaluations of the cohort took place at our research center. In face-to-face interviews at baseline and follow-up evaluations, information was collected using structured questionnaires concerning the child's and mother's health. Family medical history and data on demographic, socioeconomic and psychosocial circumstances, and lifestyles were also collected. A physical examination including anthropometric assessment was performed to children and mothers. Routine primary care data regarding the child's development were periodically extracted from the Child's Health Book. Velocities of growth in weight and length/height, across different age periods, were obtained through multilevel linear spline models. Weight trajectories since birth up to the age of 6 years were identified through normal mixture modeling for model-based clustering.

In each evaluation, a child blood sample drawn from an antecubital vein, after overnight fasting, were collected and, at 7 years of age, a subsample of 400 participants was selected to measure the following bone metabolism markers: total alkaline phosphatase (tALP), osteocalcin (OC) and β -isomerized C-terminal telopeptides of type I collagen (β -CTX).

In the 7 years-old follow-up evaluation a consecutive subsample of children who attended the face-to-face interview was invited to undergo a whole body dual-energy X-ray absorptiometry (DXA). Scanning was successfully performed in 2408 children (79.9% of the eligible sample and 27.8% of the entire cohort). Bone mineral content (BMC) and areal bone mineral density (aBMD) were obtained from DXA scan and a measure of size-corrected BMC (scBMC) was calculated.

Results

Paper I

In 2167 mother-child pairs, 35.8% of the mothers were overweight or obese [body mass index (BMI) ≥ 25 kg/m²] at the beginning of pregnancy. According to Institute of Medicine (IOM) recommendations for gestational weight gain, we found that 36.6% mothers gained excessive weight during pregnancy. We estimated heterogeneous associations between GWG and bone mineralization in the offspring at 7 years of age according to maternal BMI. Among under/normal weight mothers there was a positive association between GWG and offspring bone measures: per 5 kg of GWG, BMC: 0.07 standard deviation (SD) (95% confidence interval - 95% CI: 0.01, 0.12); aBMD: 0.10 SD (95% CI: 0.05, 0.15), while in overweight/obese mothers no effect of GWG on bone was observed: BMC: 0.02 SD (95% CI: -0.04, 0.09); aBMD: 0.02 SD (95% CI: -0.04, 0.08). When GWG was analyzed using IOM recommended categories, there were no differences in offspring mean bone properties between mothers gaining excessive and adequate weight during pregnancy, for both early pregnancy BMI groups.

Paper II

In 1853 children, all the five weight velocities ("early neonatal": 0-10 days, "early infancy": 10 days-3 months, "late infancy": 3-12 months, "early childhood": 1-3 years, and "later childhood": 3-6 years) and four length/height velocities ("early infancy": 0-3 months, "late infancy": 3-12 months, "early childhood": 1-3 years, and "later childhood": 3-6 years) were associated with increased bone mass and height at 7 years. Strongest associations were observed for growth in early childhood [(per 1 SD increase in weight velocity height-adjusted BMC z-score increased by 0.27 (95% CI: 0.22, 0.32) in girls and 0.24 (95% CI: 0.19, 0.29) in boys; per 1 SD increase in length/height velocity height-adjusted BMC z-score increased by 0.12 (95% CI: 0.07, 0.17) in girls and 0.11 (95% CI: 0.06, 0.16) in boys]. Estimates remained positive, albeit attenuated, after adjusting for preceding velocities of growth.

Paper III

In 1889 7-year-old children, 66.2% had a trajectory of growth characterized by “normal weight gain”, 8.4% had a “weight gain during infancy” trajectory, 14.6% had a “weight gain during childhood” trajectory and 10.9% had a “persistent weight gain” trajectory. Compared with the “normal weight gain” group, bone measures were greater in all the remaining trajectories. Children who followed a “persistent weight gain” trajectory since birth had clearly increased bone mass and density in comparison to “normal weight gain” children (girls [BMC: 674.0 vs. 559.8 g, aBMD: 0.677 vs. 0.588 g/cm², scBMC: 640.7 vs. 577.4 g], boys [BMC: 689.4 vs. 580.8 g, aBMD: 0.682 vs. 0.611 g/cm², scBMC: 633.0 vs. 595.6 g]). After adjustment for current weight, girls with a “weight gain during childhood” trajectory had greater bone measures than those with a “normal weight gain” trajectory (BMC: 601.4 vs. 589.2 g, aBMD: 0.618 vs. 0.609 g/cm²).

Paper IV

In 395 children, reference intervals (2.5th- 97.5th percentile) for serum tALP was 159 - 439 U/l; for β -CTx was 470 - 1690 ng/l and for OC was 52.5 - 137.7 μ g/l in girls and 50.0 - 129.9 μ g/l in boys. We found a moderate correlation between serum concentrations of bone-specific metabolism markers OC and β -CTx, likely representing the dynamic nature of bone turnover. Concentration of tALP increased slightly with height (Pearson partial correlations (r_{partial}) controlled for sex=0.26, 95% CI: 0.17, 0.35), was higher in overweight than in healthy weight children, and in children who gained weight above average during infancy. No correlations were found between OC or β -CTx and growth. In girls, OC was slightly correlated with measures of total body less head BMC (r_{partial} =0.22, 95% CI: 0.08, 0.35), subtotal aBMD (r_{partial} =0.20, 95% CI: 0.06, 0.33) and lumbar spine aBMD (r_{partial} =0.23, 95% CI: 0.09, 0.36). tALP and β -CTx were not correlated with any of the DXA-derived bone measures. Therefore, bone formation and resorption markers seemed to have limited usefulness to describe overall anthropometric growth and bone mineralization status in generally healthy children.

Using association between socioeconomic position (SEP) and fat mass as a benchmark, in a subsample of 2408 children, we found that among 10 SEP indicators associated with fat mass only educational level and occupational level of caregivers was associated with BMC: [educational level of the main (less than higher vs. higher education, standardized linear regression coefficient: 0.15, 95% CI: 0.07, 0.24) and secondary (0.13, 95% CI: 0.03, 0.22) caregivers; occupations of the main (blue and lower white vs. upper white collar: 0.12, 95% CI: 0.03, 0.20) and secondary (0.10, 95% CI: 0.01, 0.18) caregivers]. A similar pattern to that observed for BMC was found for lean mass and height. In summary, assuming that traits that are causally related share causes to a measurable extent, the observation that fat mass and BMC did not share socioeconomic causes, may mean that fat mass has a small specific effect on BMC in childhood beyond its contribution to body size.

Conclusions

In the present study, we found no beneficial effect of excessive maternal GWG on childhood bone mineralization. Therefore, adherence to current recommendations for pregnancy weight gain may contribute to optimize child's skeletal health. Regarding early life growth, our findings suggested that second and third years of life might represent a sensitive period for the effect of growth in height and weight on childhood bone mass, partly through their effect on concurrent body size. Additionally, the shape of the overall trajectory of weight gain since birth up to 6 years was associated with DXA-derived bone measures, but not with bone turnover, at 7 years of age. Markers of bone turnover were also not particularly related to bone mineralization suggesting their limited usefulness in generally healthy children. Finally, we found that fat and bone mass did not share socioeconomic causes, from which we concluded that adiposity is likely to have a small specific effect on bone mass in childhood beyond its contribution to body size.

Resumo

Introdução

As fraturas de fragilidade estão associadas a elevada morbidade e mortalidade e constituem um importante problema de saúde pública associado a custos substanciais para os doentes e para a sociedade. Um dos principais fatores modificáveis que determinam o risco de fratura é a resistência óssea que depende do tamanho, morfologia e propriedades materiais do osso. Como em muitas outras doenças crônicas, a abordagem teórica atualmente utilizada para explicar a etiologia do desenvolvimento de pior resistência óssea, e subsequente risco de fratura, baseia-se na premissa de que a qualidade óssea é em grande parte conservada desde as primeiras décadas de vida, e modulada por interações gene-ambiente. A corpulência é reconhecida como um dos principais determinantes da fragilidade óssea, e têm sido encontradas fortes relações transversais entre o tamanho e composição corporal e as propriedades do osso desde idades pediátricas. No entanto, permanecem por esclarecer algumas questões importantes sobre os efeitos longitudinais do crescimento no início da vida na mineralização óssea das crianças. Nomeadamente, não é claro o efeito do ganho de peso gestacional (GPG) da mãe nas propriedades físicas do osso da criança. Além disso, desconhece-se em que medida o conhecimento das trajetórias de crescimento desde o nascimento melhora a predição estatística das medidas de mineralização ou do *turnover* ósseo durante a primeira década de vida. Finalmente, permanece por esclarecer o potencial específico da adiposidade para influenciar as propriedades físicas do osso em crianças.

Objetivos

Os objetivos do presente trabalho foram:

1. Quantificar as associações entre o ganho de peso gestacional e a mineralização óssea da criança, testando o índice de massa corporal no início da gravidez como modificador de efeito (Artigo I)
2. Caracterizar o impacto do crescimento no início da vida na mineralização óssea na infância através da:

- a. Identificação de períodos sensíveis para o efeito do crescimento no início da vida na mineralização óssea na infância (Artigo II)
 - b. Determinação do efeito de diferentes trajetórias de ganho de peso desde o nascimento na mineralização óssea na infância (Artigo III)
3. Descrever marcadores de remodelação óssea em crianças globalmente saudáveis e determinar as suas correlações com o crescimento antropométrico e a mineralização óssea (Artigo IV)
4. Testar o potencial específico da adiposidade para influenciar a massa óssea em crianças, usando causas comuns como um *benchmark* (Artigo V)

Métodos

Foram utilizados dados do estudo Geração XXI, a primeira coorte de nascimentos portuguesa realizado no Porto. Foram consideradas elegíveis para o estudo, todas as mulheres que tiveram partos originando nados-vivos com idade gestacional superior a 23 semanas numa das cinco maternidades de nível III do Porto, entre abril de 2005 e agosto de 2006, e que residiam na área de referência das maternidades. Das mães elegíveis, 70% foram convidadas e, destas, 91,4% aceitaram participar. Todas as 8647 crianças incluídas no estudo ao nascimento foram convidadas a participar em reavaliações da coorte aos quatro (entre 2009 e 2011) e sete (entre 2012 e 2014) anos (86,3% e 79,7% de participação, respetivamente).

No recrutamento, que ocorreu nas primeiras 24 a 72 horas após o parto, entrevistadores treinados foram responsáveis por apresentar o estudo e convidar as mães. As reavaliações da coorte ocorreram no nosso departamento de investigação. Ao nascimento e nas reavaliações subsequentes foram realizadas entrevistas presenciais, utilizando questionários estruturados, com o objetivo de recolher informação sobre a saúde da criança da mãe. Foi também recolhida informação sobre a história familiar de doença, características demográficas, socioeconómicas e psicossociais, e estilos de vida. Foi também realizado um exame físico que incluía uma avaliação antropométrica da criança e da mãe. Foram extraídos do Boletim de Saúde Infantil, dados de rotina dos cuidados primários relativos ao crescimento e desenvolvimento da criança. As velocidades de crescimento em peso e comprimento/altura, em diferentes intervalos de idade, foram obtidas através de modelos lineares multinível usando

splines. As trajetórias de peso do nascimento até aos 6 anos de idade foram identificadas através de modelação de mistura normal para *clustering* baseado em modelos.

Em cada avaliação, foi colhida uma amostra de sangue de uma veia antecubital da criança, após jejum noturno e, aos 7 anos de idade, foi selecionada uma subamostra de 400 participantes para determinação dos seguintes marcadores de metabolismo ósseo: fosfatase alcalina total (tALP), osteocalcina (OC) e telopeptídeos β -isomerizados do C-terminal do colagénio tipo I (β -CTX).

Na reavaliação da coorte aos 7 anos de idade, uma subamostra consecutiva de crianças que compareceu à entrevista presencial foi convidada a fazer um exame de absorciometria de raio-X de dupla energia (DXA). O exame foi realizado com sucesso em 2408 crianças (79,9% da amostra elegível e 27,8% de toda a coorte). O conteúdo mineral ósseo (CMO) e a densidade mineral óssea areal (DMOa) foram obtidos a partir do exame DXA e calculou-se uma medida de BMC corrigida para a corpulência (DMOcc).

Resultados

Artigo I

Em 2167 pares mãe-filho, 35,8% das mães apresentavam sobrepeso ou obesidade [índice de massa corporal (IMC) ≥ 25 kg/m²] no início da gravidez. Usando como referência as recomendações do *Institute of Medicine* (IOM) para ganho de peso gestacional, observou-se que 36,6% das mulheres tinham ganho peso excessivo durante a gravidez. Observaram-se relações heterogêneas entre GPG e mineralização óssea da criança aos 7 anos de acordo com o IMC da mãe. Nas mães com peso baixo/normal observou-se uma associação positiva entre GPG e as propriedades ósseas da criança: por 5 kg de GPG, CMO: 0.07 desvios-padrão (DP) (intervalo de confiança a 95% - IC 95%: 0,01; 0,12); DMOa: 0.10 DP (IC 95%: 0,05; 0,15), enquanto em mães com sobrepeso/obesidade não foi observado efeito do GPG no osso: CMO: 0.02 DP (IC 95%: -0,04; 0,09); DMOa: 0.02 DP (IC 95%: -0,04; 0,08). Quando o GPG foi analisado usando as categorias de referência do IOM, não se observaram diferença nas médias das propriedades ósseas da criança, entre mães que tinham ganho peso excessivo e as que tinham ganho peso adequado durante a gravidez, para ambos os grupos de IMC no início da gravidez.

Artigo II

Em 1853 crianças, observou-se que todas as velocidades de peso em 5 períodos de crescimento (entre 0 e 10 dias, entre 10 dias e 3 meses, entre 3 e 12 meses, entre 1 e 3 anos e entre 3 e 6 anos), bem como as velocidades de comprimento/altura em 4 períodos de crescimento (entre 0 e 3 meses, entre 3 e 12 meses, entre 1 e 3 anos e entre 3 e 6 anos), se associaram com o aumento da massa óssea e com a altura aos 7 anos de idade. As associações mais fortes foram observadas para o crescimento entre o 1º e 3º anos de vida [(um aumento de 1 DP na velocidade de peso relacionou-se com um aumento do z-score CMO ajustado para a altura de 0,27 (IC 95%: 0,22; 0,32) nas raparigas e de 0,24 (IC 95%: 0,19; 0,29) nos rapazes; um aumento de 1 DP na velocidade de comprimento/altura relacionou-se com um aumento do z-score CMO ajustado para a altura de 0,12 (IC 95%: 0,07; 0,17) nas raparigas e 0,11 (IC 95%: 0,06; 0,16) nos rapazes].

Artigo III

Em 1889 crianças de 7 anos de idade, 66,2% tinham uma trajetória de crescimento caracterizada por “ganho normal de peso”, 8,4% tinham “maior ganho de peso no início da infância”, 14,6% tinham “maior ganho de peso mais tarde na infância” e 10,9% teve uma trajetória de “ganho persistente de peso”. Em comparação com o grupo “ganho normal de peso”, as propriedades ósseas foram significativamente maiores em todos os outros grupos de crianças nas restantes trajetórias. As crianças com uma trajetória de crescimento caracterizada por “ganho persistente de peso” desde o nascimento apresentavam significativamente mais conteúdo e densidade mineral óssea do que as crianças com “ganho normal de peso” (raparigas [CMO: 674,0 vs. 559,8 g, DMOa: 0,677 vs. 0,588 g/cm², CMOcc: 640,7 vs. 577,4 g], rapazes [CMO: 689,4 vs. 580,8 g, DMOa: 0,682 vs. 0,611 g / cm², CMOcc: 633,0 vs. 595,6 g]). Após ajuste para o peso atual, as raparigas com uma trajetória “maior ganho de peso mais tarde na infância” apresentavam melhores propriedades ósseas do que aquelas com uma trajetória de “ganho normal de peso” (CMO: 601,4 vs. 589,2 g, DMOa: 0,618 vs. 0,609 g/cm²).

Artigo IV

Em 395 crianças, os intervalos de referência para os marcadores de metabolismo ósseo analisados foram (percentil 2,5 - 97,5) - tALP sérico: 159 - 439 U/l; β -CTX: 470 - 1690 ng/l; OC: nas raparigas 52,5 - 137,7 μ g/l e nos rapazes 50,0 - 129,9 μ g/l. Identificou-se uma correlação moderada entre as concentrações séricas dos dois marcadores específicos do metabolismo ósseo, OC e β -CTX, provavelmente representando a natureza dinâmica do *turnover* ósseo. Observou-se que a concentração de tALP aumentava ligeiramente com a estatura da criança (r_{parcial} (coeficiente de correlação parcial) tendo em conta o sexo=0,26, IC 95%: 0,17; 0,35), era mais elevada em crianças com excesso de peso, relativamente a crianças de peso normal, e nas crianças com “maior ganho de peso no início da infância”. Não foram encontradas correlações entre OC ou β -CTX e variáveis de crescimento. Nas raparigas, a OC estava ligeiramente correlacionado com o CMO do corpo inteiro (r_{parcial} =0,22, IC 95% CI: 0,08; 0,35), DMOa do corpo inteiro (r_{parcial} =0,20, IC 95% CI: 0,06; 0,33) e a DMOa da coluna lombar (r_{parcial} =0,23, IC 95% CI: 0,09; 0,36). As concentrações séricas de tALP e β -CTX não estavam correlacionados com nenhuma das propriedades ósseas obtidas por DXA. Estes resultados sugerem que os marcadores de formação e reabsorção óssea em crianças na sua maioria saudáveis tem utilidade limitada para descrever quer o crescimento antropométrico quer a mineralização óssea.

Artigo V

Usando a associação entre a posição socioeconómica (PSE) e massa gorda como *benchmark*, observou-se que, numa subamostra de 2408 crianças, dos 10 indicadores de PSE associados à massa gorda, apenas a escolaridade e a ocupação dos cuidadores estavam associados ao CMO: coeficiente de regressão linear estandardizado e respetivos IC 95% para escolaridade do principal cuidador [menor que escolaridade superior vs. escolaridade superior, 0,15 (IC 95%: 0,07; 0,24)]; escolaridade do cuidador secundário [0,13 (IC 95%: 0,03; 0,22)]; ocupação do cuidador principal [ocupações de manuais vs. não manuais, 0,12 (IC 95%: 0,03; 0,20)]; ocupação do cuidador secundário [0,10 (IC 95%: 0,01; 0,18)]. Foi encontrado um padrão semelhante ao observado para o CMO para massa magra e estatura. Em suma, supondo que variáveis que têm uma relação causal entre si também partilham determinantes, o facto de não se observarem as mesmas causas socioeconómicas para a massa gorda e CMO, pode

significar que a massa gorda tem um pequeno efeito específico sobre o CMO da criança além da sua contribuição para a corpulência.

Conclusões

No presente estudo, não se observou efeito benéfico do GPG excessivo da mãe na mineralização óssea da criança. Assim, a adesão às recomendações atuais para ganho de peso na gravidez pode contribuir para otimizar a saúde óssea da criança. Em relação ao crescimento no início da vida, os resultados sugeriram que o segundo e o terceiro anos de vida podem representar um período sensível para o efeito do crescimento em altura e em peso na massa óssea da criança, parcialmente através do efeito na corpulência atual. Além disso, a forma global da trajetória de ganho de peso desde o nascimento até aos 6 anos associou-se às propriedades ósseas obtidas por DXA, mas não aos marcadores do *turnover* ósseo, aos 7 anos de idade. Estes marcadores também não se mostraram estar particularmente relacionados com a mineralização óssea, o que pode sugerir que a sua utilidade é limitada quando se refere a crianças saudáveis. Finalmente, observou-se que a massa gorda e massa óssea não partilhavam causas socioeconómicas, pelo que se concluiu que a adiposidade tem provavelmente pouco efeito específico na massa óssea na infância, para além da sua contribuição para a corpulência.

1. Introduction

1.1. The burden of musculoskeletal disorders

Musculoskeletal conditions comprise a group of more than 150 diseases and syndromes, being diverse with regards to pathophysiology, but linked anatomically, and by their association with pain and loss of physical function [1]. Historically, musculoskeletal disorders have been underestimated, mainly due to their low fatality rate and were viewed as irreversible conditions or simply part of the ageing process [2]. However, nowadays, it is recognized that this group of diseases has an enormous and growing impact on populations worldwide, being the largest contributor to disability in high-income countries [3]. In the 2017 Global Burden of Disease Study, musculoskeletal disorders were the major contributor to the global number of years of life lost due to disability (YLDs), being responsible for 15.9% of the total YLDs, which reflected an increase of 66.0% in the number of YLDs since 1990 and of 20.0% since 2007 [4].

Severe long-term pain, impaired mobility and function, and reduced quality of life and mental well-being, affecting the ability to work and actively participate in all aspects of life, are the most common consequences of musculoskeletal conditions [5]. Therefore, musculoskeletal conditions have a profound economic impact, both for the individual and society, through direct health expenditure and indirect costs related to loss of productivity [6].

The prevalence and burden of musculoskeletal conditions are predicted to rise as the population grows and ages, and the prevalence of risk factors for noncommunicable diseases (such as obesity and sedentary lifestyles) increases, particularly in developing countries [7].

Among musculoskeletal conditions, osteoporosis, a skeletal disorder characterized by low bone mass, deterioration of the bone tissue microarchitecture and compromised bone strength, contributes largely to this burden by increasing the risk of low-trauma fracture [2]. Fragility fractures are associated with chronic pain, reduced mobility and disability, and an increasing degree of dependence, thus representing the most important outcome of bone fragility, and their prevention is an essential goal for the early promotion of bone health.

1.2. The population burden of fragility fractures

In the general population, fracture incidence has a bimodal age distribution peaking during childhood and in the elderly population (Figure 1) [8,9]. This pattern of fracture incidence across the age spectrum results from the relative bone fragility observed in these two periods of life, due to skeletal immaturity (and partly also to increased trauma frequency and severity) in childhood and to the age-related structural deterioration of the skeleton in the elderly [10,11].

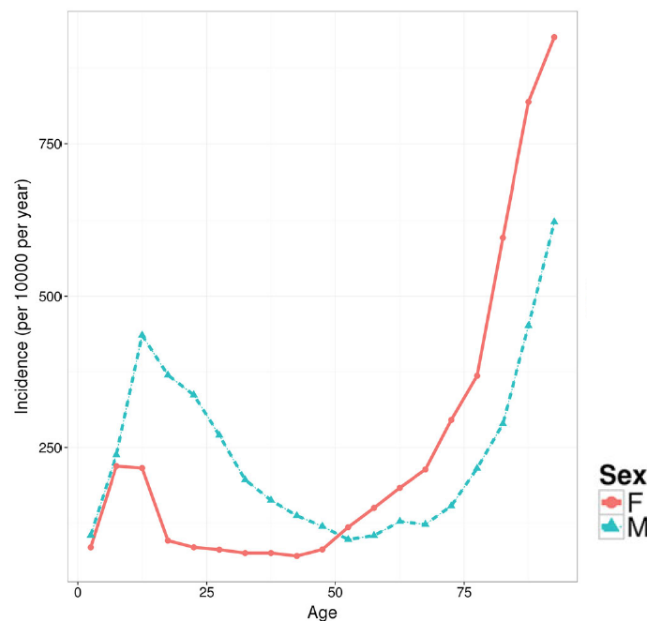


Figure 1. Annual fracture incidence (at all fracture sites) per 10 000 people, by age and sex, in the Australian general population 2006-2007 (reproduced from Pasco *et al.* [8])

In adulthood, fracture incidence increases with age as a function of age-related worsening of the intrinsic physical properties of bone tissue and due to a greater propensity to fall [12]. The incidence of falls increases with age because several sensory systems that control posture (vestibular, visual, and somatosensory) become compromised with advancing age. In addition, muscle mass, which prevents instability and corrects imbalance, declines 3 to 8% per decade from age 30 and on, and a greater decline is observed after age 65 [13,14].

As illustrated in Figure 2, different types of fragility fractures, with different impacts on the patients who suffer them, are likely to occur at different stages of life. Overall, as age advances, the prognosis of fracture worsens, due to a generally debilitated health status and to the presence of co-morbidities [15,16].

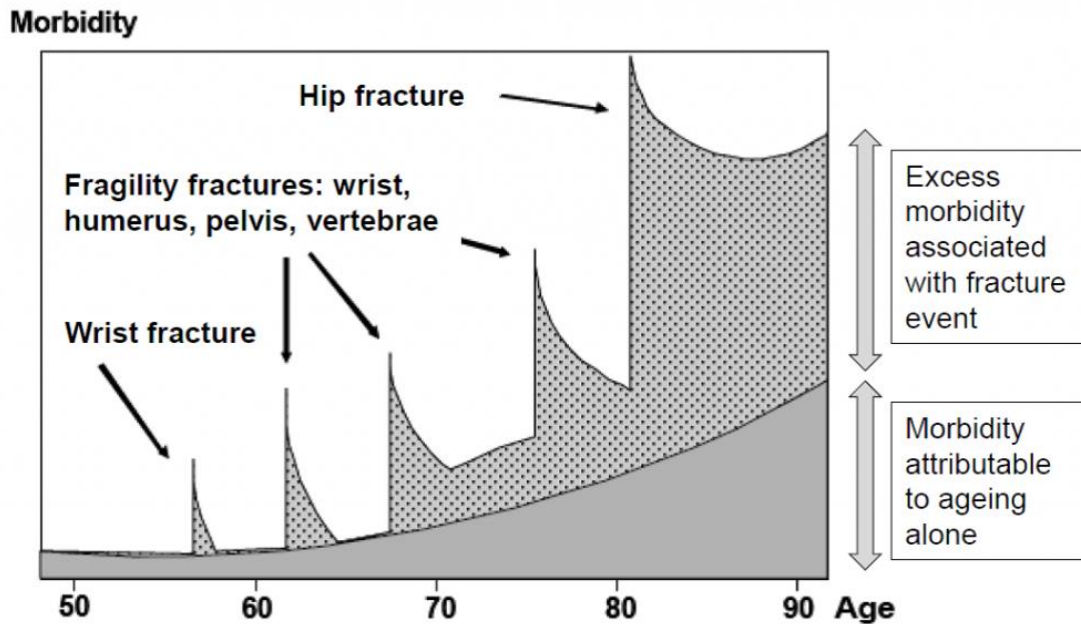


Figure 2. Schematic representation of the morbidity associated with different fractures with age (adapted and reproduced from Bone Health and Osteoporosis: A Report of the Surgeon General [15])

Fragility fractures are defined as fractures that result from a low-energy trauma, such as a fall from a standing height or from stairs, steps or curbs, or from moderate trauma, other than a fall, such as a collision with an object during daily routine [17]. Alternatively, as fractures are particularly frequent in older ages, they can also be operationalized as those fractures that occur at a skeletal site with decreased bone strength and whose incidence increases after the age of 50 years [18]. The anatomical sites where most fractures due to skeletal fragility are likely to occur are, by order of the related disability burden, the hip, the lumbar spine and the distal forearm [18,19]. Since hip fractures are typically related with decreased bone strength, leading almost always to hospital admission and resulting in major temporary or even permanent functional disability, they have become the standard outcome for estimating the burden of bone fragility. These fractures also contribute for the largest proportion of the morbidity, mortality and costs attributable to fragility fractures [1,20].

Besides the age effect observed in fracture trends, a gender effect in the susceptibility to fractures has been described, with women having an increased fracture risk compared to men. In different population settings, the lifetime risk of fracture was estimated to be two to four times higher in women than in men [21-25]. Differences in fracture risk between sexes are attributable

not only to higher areal bone mineral density (BMD) in men than in women, but also to increased bone size and bone geometry resulting in higher bone strength, and greater mechanical resistance of the male skeleton throughout life [21,26]. Also, relevant loss of bone mass occurs among postmenopausal women [26]. Nevertheless, even though the mortality rate after hip fracture is described to be higher in men than in women, the huge morbidity and financial burden of fragility fractures in the population arise mainly from fractures occurring in women [27].

Worldwide, but particularly in high-income countries, age-related fragility fractures are frequent events. In 2000, there were an estimated 9.0 million fragility fractures occurring worldwide of which, 1.6 million were at the hip, 1.7 million at the forearm and 1.4 million were clinical vertebral fractures [28]. In Europe, where fragility fractures account for over one third of all those occurring worldwide, the number of new fractures, in 2010, was estimated to be 3.5 million and included 610,000 hip fractures [29]. More recently, a report from the International Osteoporosis Foundation (IOF) referred that, in 2017, there were an estimated 2.7 million fractures occurring in six European countries (France, Germany, Italy, Spain, Sweden, and United Kingdom) of which, 51% were major fragility fractures (hip, spine, humerus, or forearm fractures) [30].

A substantial variation in the estimates of incidence rates of fragility fractures by geographic region is supported by numerous country-specific and regional studies (Figure 3) [25,29,31,32]. Excluding countries providing very old or poor-quality estimates, age-standardized hip fracture rates vary around 10-fold between countries [31,32]. Median age-standardized hip fracture rates are the highest in Western populations (North America and Europe) followed by Oceania, Asia, Middle East, Oceania, Latin America and Africa [25]. Annual hip fracture incidence rates higher than 250/100,000 have been described in Denmark, Norway, Sweden and Austria, while the lowest rates (<150/100,000) were found in Nigeria, South Africa, Tunisia and Ecuador [31]. Nevertheless, some high-risk countries (e.g., Malta, Argentina, Singapore and Taiwan) seem to escape this pattern [31]. Within the same continent, rates also seem to vary markedly: in Europe, for example, Scandinavia presents some of the highest age-adjusted rates of hip fractures worldwide, almost seven-fold higher than in southern European countries [25,31]. Furthermore, hip fracture incidence rates seem to be lower in rural than in urban areas [33].

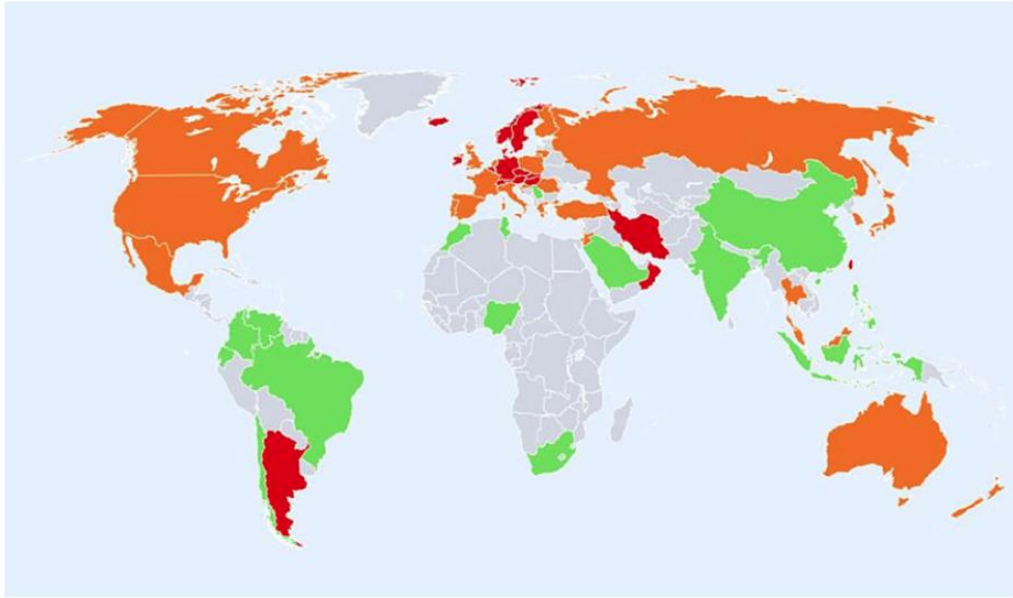
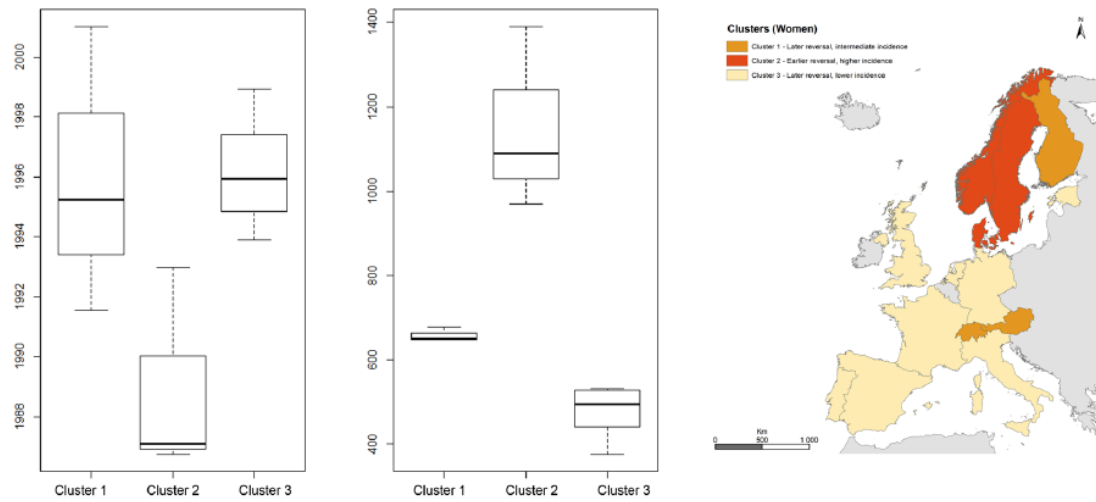


Figure 3. Hip fracture rates for men and women combined in different countries of the world, categorized by risk. Where estimates are available, countries are color coded red (annual incidence >250/100,000), orange (150–250/100,000) or green (<150/100,000) (reproduced from Kanis *et al.*[31])

In addition to the large geographic variation in the incidence of hip fractures throughout the world, contrasting secular trends were described across different countries and regions [34–36]. Temporal trends in the age- and sex-adjusted incidence of hip fractures worldwide reveal that, in high-income countries, after increases throughout the second half of the 20th century, incidence rates seem to have stabilized, or started to decline, over the last two decades [34]. Indeed, in Europe specifically, hip fracture incidence rates in different countries can be described within the same overall pattern of secular trend reversal, with significant intercountry variability in the timing of reversal and peak fracture rates, which suggests that European countries show variations of the same hip fracture epidemic (Figure 4) [37]. In developing populations, however, particularly in Latin America and Asia, there is evidence for a continuous rise in age-adjusted hip fracture rates [34,35]. Although time trends for fragility fractures at other anatomical sites are less well characterized, similar trends to those described for hip fractures have been suggested [35,36].

a) Women



b) Men

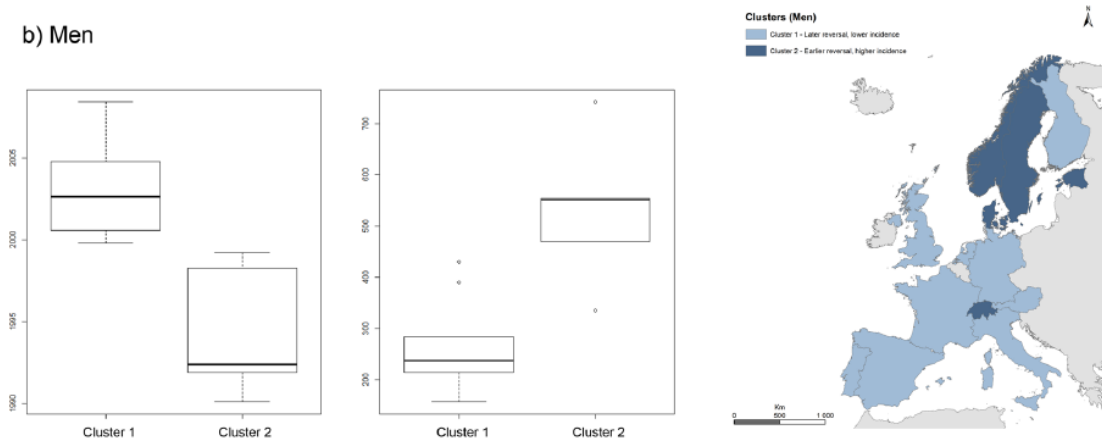


Figure 4. Distribution of calendar year of trend reversal (boxplots on the left) and peak incidence rates (boxplots on the right) in country clusters and the geographical distribution of countries in each cluster (reproduced from Lucas *et al.* [37])

As the incidence of osteoporosis and associated fractures increases exponentially with advancing age [38], the burden of fragility fractures is expected to increase, particularly in developing countries [39]. Based on current demographic changes and if age-adjusted incidence rates for hip fractures remain stable, it is estimated that the global annual number of hip fractures may reach 6.3 million by 2050 [40]. Additionally, since fracture rates seem to be rising in many parts of the world, the projected number of hip fractures worldwide could be as high as 8.2 million by 2050, if a 1% per year rise in age-adjusted rates is assumed [40]. The percentage increase will be greater in men (310%) than in women (240%) [41]. In Europe, the

number of individuals with osteoporosis is projected to increase from 27.5 million in 2010 to 33.9 million in 2025, which translates into an estimated annual number of 4.5 million incident fragility fractures [29]. Thus, fractures will continue to place a high financial burden on individuals, healthcare systems, and ultimately on the society.

As a public health issue, hip fractures are associated with important short- and long-term morbidity, with a significant impact on quality of life, as well as increased mortality [42,43]. Hip fracture survivors experience worse mobility, health and quality of life, less independence in function, and higher rates of institutionalization than age matched control subjects. It has been estimated that, after a fragility fracture, 50% of previously independent people do not return to their pre-fracture level of autonomy, 30 to 50% of patients become totally dependent, and 10 to 20% of patients are institutionalized within 6 to 12 months post-fracture [28,44].

The burden of disease due to incident hip fracture using disability-adjusted life-years (DALYs) was estimated to be 1.75 million DALYs lost globally, in 1990, due to hip fractures, which translates into an average loss of 0.1% of the total burden of disease [36,45]. Recently, using real-life data of a large cohort consortium of middle-aged and older individuals in Europe and the United States followed for up 13 years, the burden of disease due to hip fracture was 5964 DALYs (27 per 1000 individuals), representing a loss of 2.7% in the healthy life expectancy in this population [46]. In comparative terms, fragility fractures account for more DALYs than most common cancers in Europe [28].

Despite advances in the management of osteoporosis, fragility fractures, especially hip fractures, are associated with a higher mortality rate [43,47]. Around 20 to 40% of people die within the first year following a hip fracture event [34]. In comparison with general population mortality rates, hip fractures are associated with a two- to four-fold increased risk of mortality during the first year after the event, in women and men respectively [48].

Economically, mainly due to their high frequency and associated incapacity, fragility fractures pose a significant economic burden to the individual, to financial and health systems, and ultimately to societies. In a recent systematic review of studies from 27 countries reporting costs attributable to hip fractures, the average cost of the index hospitalization following a hip fracture was estimated to be USD 10,075. Additionally, the health and social care costs in the 12 months following hip fractures were estimated to be USD 43,669 [49]. In fact, expenses of hospital care and rehabilitation during the first year following hip fractures exceed those of other highly prevalent pathologies of the elderly, such as acute coronary syndrome and ischemic

stroke [49]. Hence, in the United States, the annual estimated cost attributable to incident and prevalent fractures was more than USD 19 billion in 2005 and it is speculated that it will increase up to USD 25.3 billion by 2025 [50]. In Europe, the total monetary osteoporosis burden in 2010 was estimated at EUR 37 billion annually [29]. Costs attributable to the treatment of incident fractures accounted for 66% of this cost, while pharmacological prevention and long-term fracture care represented 5% and 29% of the total costs, respectively [29]. Furthermore, if the costs attributable to the quality-adjusted life years lost were included in the cost associated with fracture management, estimates would be EUR 98 billion and have been projected to increase around 25% between 2010 and 2025, up to EUR 121 billion by 2025 [29].

1.3. Bone fragility and fractures

Fractures depend upon both bone strength and propensity to trauma (Figure 5) [51]. From a mechanical perspective, fractures represent a structural failure of the bone, which occurs when the loads applied to the bone exceed its strength. The forces applied to the bone will depend on the specific activity, and will vary with the magnitude and direction of the loads applied to it. For example, during a fall, the loads applied to the proximal femur depend on the height and direction of the fall, the impact surface, the extent of the natural “trochanteric padding” i.e. of the soft tissue overlying the hip, and the ability of the individual to decrease the impact of the fall [51].

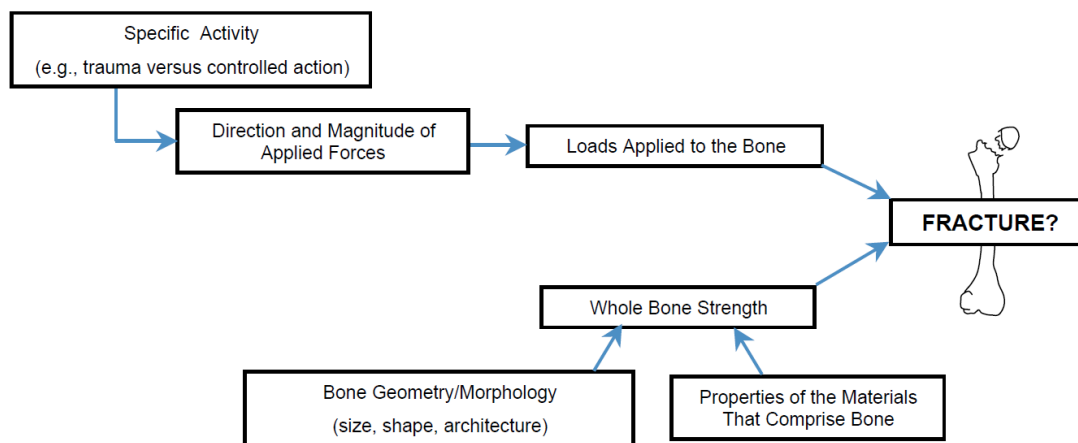


Figure 5. Etiology of age-related fractures (reproduced from Bouxsein [51])

Whole bone strength, i.e. the load-bearing capacity of a bone and its consequent ability to resist fracture, depends on the bone’s biomechanical properties. These include structural properties,

which are influenced by the amount of bone (i.e. size or mass) and the spatial distribution of bone tissue (i.e. shape and architecture) and material properties, which reflect the intrinsic biomechanical characteristics of bone tissue (apparent density and microstructural arrangement of trabeculae) [51] (Figure 6). Therefore, bone fragility is said to occur when the biomechanical properties of the organ as a whole are compromised, leading to increased fracture risk [52].

Maintenance of the shape, mass and size of the skeleton depends on the continuous process of remodeling. Bone remodeling is a process characterized by the coordinated actions of osteoclasts and osteoblasts, organized in bone multicellular units, following an activation-resorption-formation sequence of events [53,54]. This process involves the removal of mineralized bone by osteoclasts, followed by the replacement with newly formed bone matrix (osteoid) by osteoblasts, which then undergoes mineralization to form new bone. Bone remodeling takes place in four different bone surfaces, namely, periosteal, endocortical, trabecular and intracortical (or Haversian). Within each envelope, osteoclastic bone resorption is tightly coupled with local osteoblastic bone formation, and the balance between these two processes allows bone to adapt structure to function in response to stress and other biomechanical forces. Also, bone remodeling is an integral part of the calcium homeostatic system, and provides a crucial mechanism for the removal of old bone and repair of microdamages in bone matrix [62]. Contrasting to bone modeling, that is the predominant process occurring in childhood, bone remodeling predominates in adulthood, and is thus essential for the maintenance of overall bone strength and for repairing damaged tissue throughout the life course [55].

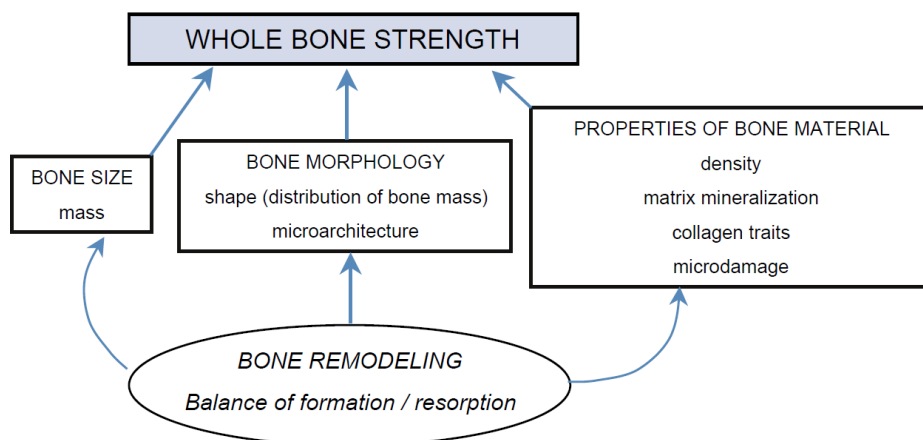


Figure 6. Determinants of whole bone strength (reproduced from Bouxsein [51])

1.4. Estimating bone fragility - Bone mass as a surrogate for bone strength

According to the United States National Institutes of Health Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy, from 2001, osteoporosis was defined as “a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture” [19]. As previously mentioned, whole bone strength depends on the combination of the amount of bone (i.e. size), the spatial distribution of the bone mass (i.e. shape), and the intrinsic properties of the materials that comprise the bone. Among these, emphasis has been placed on the mass component as it is easily quantifiable without the need for invasive testing and it is, to a certain extent, modifiable through interventions [15]. The importance of bone mass as a determinant of bone strength is reflected in fact in the World Health Organization (WHO) operational definition of osteoporosis [56]. This definition established four thresholds based on reference populations of healthy young women to be used in diagnosing osteoporosis and determining intervention; a BMD value higher than 1 standard deviation (SD) below the young adult female reference mean (T-score ≥ -1 SD) indicates normal bone density, while a BMD value between 1 and 2.5 SD below the reference mean (T-score < -1 and > -2.5 SD) is indicative of *Osteopenia*, a BMD value 2.5 (SD) or more below the (T-score ≤ -2.5 SD) reflects *Osteoporosis* and a BMD value 2.5 SD or more below the reference mean in the presence of 1 or more fragility fractures indicates *Severe osteoporosis (established osteoporosis)*. More recently, in 2008, dual-energy X-ray absorptiometry (DXA)-derived BMD measurements at the femoral neck were recommended as the reference standard for the description of osteoporosis [57]. A normative database of femoral neck measurements obtained in women aged 20 to 29 years, as part of the NHANES III, should be used as the reference data [57].

The development of DXA in the mid-1980s, as a rapid and safe imaging modality, allowed for the measurement of bone mineral mass. DXA distinguishes bone mass from soft tissue, both for the total body and for specific regions, by the differential attenuation of the high and low energy X-rays of these tissues. This technique uses an X-ray source to produce a beam of two discrete energies, and while bone tissue, mainly constituted of phosphorus and calcium, attenuates high-energy photons, soft tissue composed by muscle, fat, skin and water attenuates low-energy photons. In addition to the estimation of the content of bone mass and projected bone area, DXA also provides a measure of bone quality, or BMD, that is computed as the ratio between bone mass and bone area [58].

Considering the high precision and accuracy of DXA, which make this technique the standard method to measure BMD in order to assess fracture risk, other advantages include very low radiation dose exposure to patients, availability, short scan times, ease of use and a relatively low cost [59]. DXA is also the method of choice to assess bone mass in children, however errors related to growth and maturity significantly diminish the accuracy of this method for bone quality measurement [60,61].

The importance of the identification of osteoporosis is mainly chiefly with regard to associated fractures and their sequelae [20,62]. In prospective studies among adults, the risk of fragility fracture increases continuously as BMD declines by a factor of 1.5–3.0 for each SD decrease in BMD [62], and similar fracture risk increments in children and adolescents have been reported [63,64]. The ability of BMD to predict fracture is comparable to the use of blood pressure to predict stroke, and significantly better than serum cholesterol to predict myocardial infarction [56]. The relationship between BMD and fracture risk was confirmed in a meta-analysis using approximately 39,000 participants from 12 cohort studies, where it was estimated that, per each SD decrease in BMD, the risk of fragility fracture increased by 1.55 (95% CI: 1.47, 1.62) and the relative risk of hip fracture increased by 2.07 (95% CI: 1.91, 2.24) [65].

However, there is increasing concern about the limitation of BMD to accurately predict bone strength and fracture risk, at the individual level, considering that the majority of fragility fractures occur in individuals with values of BMD above the threshold for osteoporosis diagnosis [66,67]. In fact, in comparison to individuals with osteoporosis, the higher number of individuals who have normal BMD, although individually at lower risk, contributes with a higher number of fractures to the total burden. For this reason, over the past years, efforts have been directed to identify clinical risk factors for the risk of fractures independently of BMD. From a series of meta-analyses using individual data from prospective population-based studies in Europe, North America, Asia and Australia, the determinants of fragility fractures identified were age, gender, body mass index, prior fragility fractures, parental history of hip fractures, long-term use of oral glucocorticoids, rheumatoid arthritis and other secondary causes of osteoporosis, current smoking and increased alcohol intake [68-72]. This consideration has led to more pragmatic approaches toward the prediction of fracture risk combining BMD with clinical and epidemiological risk factors in absolute risk calculators, such as the Fracture Risk Assessment Tool, FRAX[®] developed by the WHO [38,73]. The use of clinical risk factors in addition to BMD measurement has been demonstrated to increase the accuracy of fragility fracture (hip or major fractures) risk assessment [74,75].

1.5. The life course approach to bone health

Key stages in people's lives have particular relevance for their health and the life course approach acknowledges the importance of these stages. The growing interest in the relationship between adverse circumstances across the life course leading to changes that impact on disease risk and early mortality have led to research that explains disease etiology within a life course framework [76].

Life course theory emerged in the second half of the twentieth century in order to answer to the question of how the interrelationship of social structures, time, place and history of individuals' lives influence their own life and their successfulness [77]. Thereafter, emerging research has led to a convergence of evidence on the wide-ranging effects of early environment and the associated development for later health outcomes [78]. As the life course theory has evolved over time, four core principles have emerged from early empirical observations to provide a framework to guide research on the matter of problem identification and conceptual development. These principles are [77,79]:

- 1. Historical time and place:** the life course of individuals is embedded in and shaped by the historical times and places they experience over their lifetime;
- 2. Timing in lives:** the developmental impact of a succession of life transitions or events is contingent on when they occur in a person's life;
- 3. Linked lives:** lives are lived interdependently, and social and historical influences are expressed through this network of shared relationships;
- 4. Human agency:** individuals construct their own life course through the choices and actions they take within the opportunities and constraints of history and social circumstances.

The objective of a life course approach in epidemiology is to establish how biological, behavioral and psychosocial factors, as well as a countless number of interactions between them, operating at different stages of life and across generations, contribute to the development of adult health and disease over time [80]. Considering this approach, health outcomes in adult life may be seen as long-term effects of exposures acting from preconception through pregnancy, infancy, childhood and adolescence, through adulthood [76]. Life course epidemiology extends the developmental origins of adult disease perspective by focusing

attention on potentially sensitive periods throughout the life course [78]. Although the terms “critical period” and “sensitive period” are often used interchangeably, important distinctions exist between them. A critical period of development refers to a time window, when an exposure can have long-lasting adverse or protective effects on the structure or function of organs, tissues, or body systems, which are not modified by later experience and may eventually result in disease. Outside this window, this developmental mechanism for mediating exposure and disease risk is no longer available, i.e. an exposure in a critical period results in permanent and irreversible damage or disease. Additionally, there may also be sensitive developmental periods when exposures exert stronger effects on development than they would during other periods. It means that there is a greater scope for modification or even reversion of those changes outside the time window, than there is in a critical period [76]. Overall, both critical and sensitive periods refer to qualitatively different exposure-time interactions [81]. To understand how exposures may differentially act in critical and/or sensitive periods, a life course approach requires some understanding of physiological trajectories of normal biological systems. Most interest has focused on changing exposures, particularly developmental variables, such as height and weight, which change rapidly throughout life and may have specific periods when endogenous changes, triggered or modified by environmental factors, play a role and may have lifelong consequences on endocrine systems [78].

Adopting a life course approach presents major challenges for both the design and analysis of epidemiological studies [76]. In this context, using data sets that are suited for life course analyses, with repeated measures of exposure and/or outcomes, such as those obtained from population-based birth cohorts, are the most powerful way to examine dynamic processes and determine if any critical or sensitive periods exist [82]. For this purpose, birth cohort studies established some decades ago have been fundamental to provide relevant evidence about health and functions across the life course, and between generations that link to adult outcomes [78]. Birth cohort studies allow stating the temporal ordering of exposure variables and their inter-relationships, both directly and through intermediate variables, with the outcome measure. They allow for the operationalization of early life course exposures and conceptualization of their inter-relationships across the life course, and also to test possible pathways with potential intermediate or confounding factors [76]. Because different periods across the life course influence phases of biological development, stability or decline, longitudinal studies since birth clearly remain one of the most powerful ways to test life course models. More ambitiously, cohorts from different time periods can provide insights into whether exposure-disease

associations differ across time and how these are manifested in disease trends that are observed over time at the population level [82].

Not surprisingly, the life course theory started being used to study chronic disease distribution and its determinants, resulting in the development of a life course approach to chronic disease epidemiology [81]. This relies on a multidisciplinary framework for understanding how early- and later-life biological, behavioral and psychosocial exposures operate across an individual's life course, as well as across generations, to influence the development of chronic diseases [76]. Further, a life course approach to chronic disease epidemiology recognizes the importance of time - based on the fact that most of these conditions develop over time - and timing - based on the observation that the later effects of a particular exposure depends on the stage of life when it occurred - in understanding causal links between exposures and chronic diseases at the individual and population levels [81]. Despite recognizing the importance of early-life influences on chronic diseases, the life course approach does assume deterministic processes that preclude later-life intervention. Indeed, no adult chronic disease is likely to be explained as the predetermined outcome of exposures in utero or infancy, but rather as longer-term consequences of the complex accumulation and interaction of early and later-life exposures [81].

As with many other chronic diseases, the approach currently used to explain the etiology of suboptimal bone strength is built on the premise that there is important tracking of this characteristic throughout the life course, modulated by gene-environment interactions [83-86]. Accordingly, bone strength of an individual later in life is seen as the result of multiple influences acting throughout the whole life course. Two major aspects are thought to determine the risk of fracture in adults. One is the amount of bone mineral loss with advancing age, and the other is the maximum amount of bone mineral attained during the first decades of life - peak bone mass (PBM) [83,85,87]. PBM is the maximal amount of bone mineral accrued within bone during childhood and adolescence plus the consolidation that continues beyond the attainment of final height [88]. In light of life course epidemiology, optimizing PBM, i.e., to achieve the full genetic potential for skeletal mass during adolescence, is likely to reduce the impact of age-related bone loss later in life (Figure 7) [83,87,89].

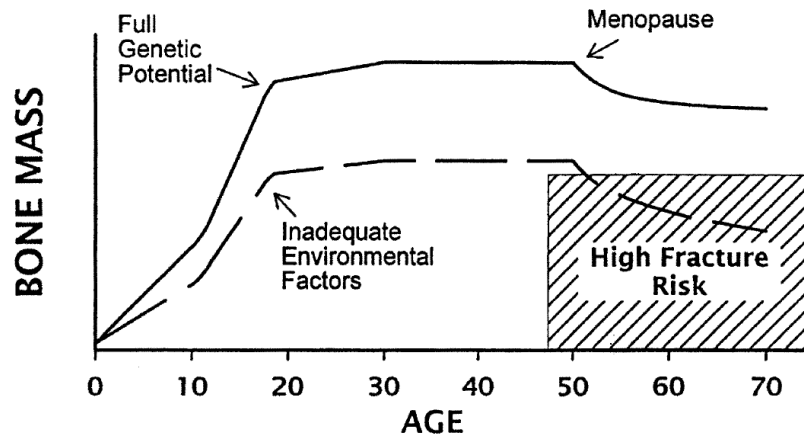


Figure 7. Diagrammatic representation of the bone mass life-line in individuals who achieve their full genetic potential for skeletal mass and in those who do not (reproduced from Heaney *et al.* [83])

In comparison to the rate of bone resorption in adulthood, bone accrual before PBM may be a stronger predictor of osteoporosis risk later in life. While a 10% increase in peak BMD was predicted to delay the development of osteoporosis by 13 years, a 10% change in the age at menopause or the rate of non-menopausal bone loss was predicted to delay osteoporosis by approximately 2 years (Figure 8) [90].

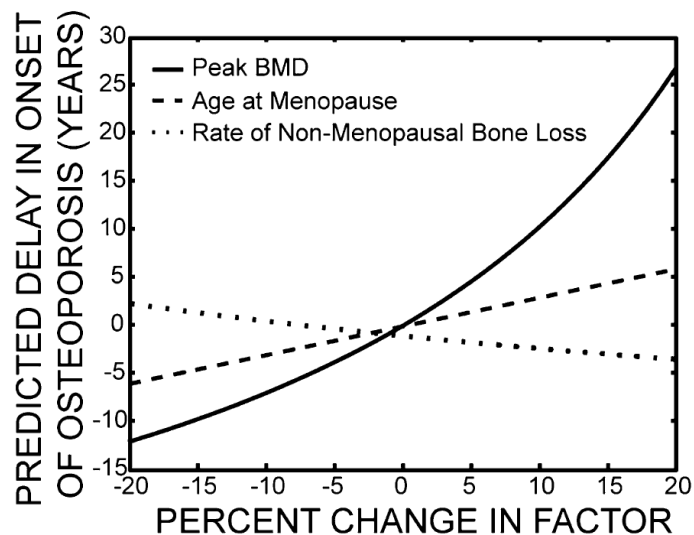


Figure 8. Delay in the development of osteoporosis predicted in response to a percent change in peak bone mass density, age at menopause or the rate of non-menopausal bone loss (reproduced from Hernandez *et al.* [90])

Therefore, as the optimization of PBM represents a potential target for interventions aimed at reducing the risk of fractures later in life, and evidence is accumulating that early environmental influences modulate PBM and fracture risk, childhood and adolescence are seen as sensitive periods for improving bone health [91,92].

In comparison to adolescence, childhood is a period characterized by a slower increase in bone mass [93]. However, as bone mass tracks from childhood, bone mass accrual throughout adolescence will be partly determined by previous bone properties [93,94]. In light of this, differences in skeletal size, shape and mass in adulthood seems to be established as early as in the first year of life [95-97]. Additionally, the relatively rapid rate of mineral gain beginning in utero and extending to the first years of life, coupled with the plasticity of skeletal development in utero, offers the possibility of profound interactions between the genome and early environment [98]. Therefore, the first years of life, specifically from fetal life until prepubertal years, are currently recognized as crucial to modifying bone traits that track throughout life [98].

1.6. Early life growth

Growth is a complex process that occurs throughout in utero development, infancy, childhood, puberty and early adulthood [99]. It comprises changes in body size as well as in body composition. Regarding linear growth during intrauterine development, the highest velocity is observed at about 18 weeks of gestational age and then slows towards the end of gestation. Sex-differences in the rate of linear growth in utero have been described with males exhibiting a more rapid linear growth than females. In the first year of life, growth velocity of total body length averages approximately 25 cm, followed by a decrease in growth to 12.5 cm in the second year. Boys grow more rapidly than girls, particularly in the first half year of life, but after 8 months of age this velocity difference decreases. Between 2 and 4 years of age, the annual height velocity decreases to 8 cm, and then to 6 cm until the age of 6 years. A plateau-like phase emerges in midchildhood, before puberty, when height velocity approaches 5.5 cm per year. During this stage, girls increase in stature slightly more rapidly than boys between 1 and 4 years but, afterwards, linear growth velocities converge in the sexes until the age of 9 years. At around 10 years old in girls and 14 years in boys, the pubertal growth spurt begins [99]. Increases in height after 12 months of age are mostly attributable to rapid longitudinal growth of the legs in comparison to the spine. Growth velocity of the legs continues to remain around twice that of the axial skeleton until puberty [98]. This implies that adverse environmental

exposures during prepuberty will lead to greater restriction of the axial skeleton, while those in infancy and puberty will produce greater deficits in appendicular growth [98].

Body weight also suffers great changes in the beginning of life [99]. In utero, body weight increases with a similar temporal pattern to growth in length, except that the maximum velocity of weight gain occurs at about 34 weeks. At around 24 weeks of gestational age, weight gain diverges between sexes, such that, at 30 to 32 weeks of gestation, boys weigh approximately 70 g more than girls. At birth, the absolute male-female weight difference is around 130 g. After birth, over the first 7 to 10 days of life, newborns lose approximately 10% of their birth weight. Neonates are expected to overcome this weight deficit within several weeks, and afterwards, during the first 3 months of life, weight gain is close to 30 g per day. Then, the mean weight gain is approximately 20, 15 and 12 g per day, in the periods between 3 and 6 months, 6 and 9 months, and 9 and 12 months, respectively. In the first year of life, the velocity of weight gain is slightly higher in males than in females, such that, at 12 months of age boys weigh approximately 10 kg and girls 9.5 kg. In the following two years, both boys and girls will gain an average of 8 g per day. Then, in midchildhood, weight gain declines to 6 to 7 g per day, and at age 7, the mean weight in boys is 23 kg, while in girls, it is 22 kg. Afterwards, during the gender-specific years of puberty, weight velocities accelerate by almost two-folds, where males gain 13.7 g per day and girls 11.5 g per day [99].

1.7. Early life bone development

Bone development is characterized by two functionally distinct phases. The first, skeletal patterning, occurs during the embryonic period and the position and shape of the various skeletal elements is established according to a genetically determined plan. The second, mineralization, takes place throughout the first decades of life up to PBM and involves a continuous process of bone growth in length and width, by longitudinal and appositional growth, respectively [88]. Skeletal formation occurs through two major mechanisms: intramembranous (in the skull and facial bones) and endochondral ossification (responsible for longitudinal growth of long bones and vertebrae). During intramembranous ossification, osteochondral progenitors differentiate directly into osteoblasts, which begin to secrete osteoid, to form membranous bone. There is no cartilage model preceding ossification in this type of bone development [98]. In contrast, during endochondral ossification that occurs at epiphyseal plates, endochondral progenitors differentiate into chondrocytes to form a cartilaginous template of the future bone. Then chondrocytes become hypertrophic, degenerate and die as a result of their matrix

calcification. Osteoprogenitor cells and blood capillaries will occupy the empty space left by the hypertrophic chondrocytes. Osteoprogenitor cells will give rise to osteoblasts, bone forming cells that will be responsible for laying down the matrix with mineralized osteoid on the surface of the calcified cartilage, increasing the size of the metaphyseal compartment. The remnants of the calcified cartilage are then resorbed by cells that break down cartilage, the chondroclasts. This process occurs continuously until the cartilage model is fully replaced by bone, meaning that the chondrocytes at growth plates exhaust their proliferative capacity, leading to the closure of the epiphyses, and cessation of linear growth [100]. In turn, appositional growth occurs through modeling, a process characterized by the uncoupling of bone formation and resorption, allowing for bone formation to occur in the periosteal surface, while resorption takes place within the endosteal surface [54]. Overall, the balance of modeling is in favor of bone formation, contributing to increases in diameter and cortical thickness. Throughout skeletal growth, bone modeling is the process responsible for substantial changes in the size and shape of bone, thereby optimizing its geometry with respect to mechanical properties [36]. The result of modeling is an altered spatial disposition of bone tissue that happens in coordination with linear growth. Bones may widen or change axis by removal or addition of bone to the appropriate surfaces by independent action of osteoblasts and osteoclasts in response to biomechanical forces [101]. There are gender-related differences in bone width, which are partly established before puberty. While, in males, cortical width increases by periosteal bone formation, in females a relatively greater endocortical apposition is observed. Consequently, males build longer and wider bones, with a slightly thicker cortex, than females [98]. Besides growth in length and modelling, bone remodeling also represents one of the major processes that determine bone mass and architecture in children. As described in the “Bone fragility and fractures” section, remodeling occurs in a coupled mechanism of formation and resorption. Once the skeleton reaches maturity, modeling rates reduce substantially but homeostatic remodeling remains active throughout the life course. [102]. From the balance between bone formation and resorption in remodeling, bone will adapt structure to function, optimizing its resistance to stress.

1.8. Factors influencing childhood bone mass accrual

Increasing evidence has shown that a complex interplay between genetic and environmental exposures, acting as early as during fetal life, shapes bone mass acquisition during childhood [92,98].

Studies in twins and families indicate that BMD is a highly heritable trait, with 50% to 80% of the variance in BMD being genetically determined [88,103]. Including populations of children and adolescents, the largest genome-wide association meta-analysis has successfully identified six adult-BMD-associated loci (WNT4, WNT16, TNFSF11, GALNT3, PTHLH and FUBP3) and a novel locus encompassing RIN3 to be robustly associated with total body BMD [104]. Although there is evidence to suggest that a substantial proportion of the variation in bone acquisition and PBM is attributable to genetic factors, current genetic markers are able to explain only a small proportion of the variation in individual bone mass [98]. Weak associations of each genetic variant with bone phenotypes are indeed expected, since there is a need for genetic mechanisms that govern key functions such as skeletal integrity to be redundant, pleiotropic and polygenic, in order to avoid perturbation triggered by the environment [105]. An evolutionary perspective of osteoporosis has described the condition as a result of increased life expectancy combined with a misadaptation to a still young bipedal status. Since bone fragility becomes apparent as a phenotype after reproductive years, there is not a clear adaptive advantage of having strong bones before and during the reproductive period. This would result in unchanged, “archaic” genes that interact with the modern environment causing a response that can lead to inefficient adaptation, i.e. bone fragility and subsequent fracture [105].

In addition to heredity, many environmental factors may act in pediatric bone growth and density including gender [106], ethnicity [107], nutrition (e.g., calcium, vitamin D, protein) [108], physical activity [109], endocrine status (e.g., sex steroids, insulin-like growth factor I) [88] and body composition [110]. Additionally, evidence has shown that fracture risk may be programmed during intrauterine life [111]. In fact, the rapid rate of mineral apposition and the plasticity of skeletal development during fetal life, make the early skeleton susceptible to interactions with environmental factors [112].

This thesis focuses on understanding the effects of anthropometric growth and body composition on childhood bone mineralization, and the following sections aim to provide a theoretical framework for the specific objectives.

1.8.1. Intrauterine influences on childhood bone mass

Environmental influences acting during intrauterine life may affect both early skeletal development and the acquisition of bone mass in childhood. Intrauterine effects impacting on childhood bone mass are largely mediated by nutrient supply to the fetus that determines fetal growth [108,113]. As an example, maternal nutrition, including maternal intake of calcium, phosphorous and protein intake, are thought to exert a direct effect on fetal skeletal growth [108]. Other mechanisms by which in utero environmental exposures can influence later child bone health include modification of gene expression by inducing epigenetic changes in the DNA [114]. Changes may also be induced by alteration in bone tissue differentiation (alteration of cell number or imbalance between cell types) or in homeostatic control mechanisms (changes in the pattern of hormonal release or tissue sensitivity to these hormones) [98].

Intrauterine life environmental variables that appear to independently influence bone growth and mineral acquisition include maternal diet, and vitamin D, the individual component most strongly associated with offspring bone development [117], maternal alcohol consumption, caffeine intake, diabetes mellitus and physical activity [88,98]. Because the majority of fetal bone is gained during the last trimester, gestational age has likewise been related with bone mass in neonates. Additionally, birth season seems to determine newborn total body mass as infants born in the winter have lower BMC than those born in the summer [88]. Maternal cigarette smoking during pregnancy has also been studied has a potential determinant of offspring bone mass but there is no consistent evidence to establish a causal relationship between fetal smoke exposure and offspring bone mass via intrauterine mechanisms [115,116]. Also, anthropometric maternal characteristics during pregnancy seem to be important determinants of offspring bone mineralization. Reduced maternal height, lower preconception maternal weight, reduced maternal fat stores during late pregnancy have all been associated with reduced whole-body BMC in children [98]. Additionally, maternal gestational weight gain during pregnancy was associated with increased bone mineral properties of offspring [117,118]. Gestational weight gain is a complex trait that reflects fetal growth but also increases in maternal tissues (e.g., blood volume, breast and uterine weight, and adipose tissue) [119]. It is the result of the physiology of pregnancy and of the pregnant women's lifestyle, in particular her diet and physical activity. Potential underlying mechanisms that explain the positive association between maternal gestational weight gain and bone mass later in life include intrauterine programming effects involving increased placental transfer of

nutrients during fetal development or a greater exposure to hormones such as leptin and estrogen [120,121]. However, in view of previous observations for a broad range of other long-term child outcomes, including body size, body composition or cardiometabolic and respiratory health, it would be likely that the positive effect of gestational weight gain on offspring bone mass would be dependent of the maternal weight status at the beginning of pregnancy [122]. Also, in light of the current recommendations for healthy gestational weight gain that are based on prepregnancy body mass index, it would be relevant to assess the impact of following these guidelines on further offspring health outcomes, other than the child health outcomes potentially related to gestational weight gain used to define the recommendations. These considerations were in fact the starting point to paper I. It is also important to emphasize that maternal gestational weight gain is an important potentially modifiable factor and a better understanding of its influence on offspring bone mineralization may be relevant to develop strategies to bone health promotion.

1.8.2. Postnatal growth-related changes of the skeleton

During childhood, due to linear growth and increases in muscle mass, the skeleton increases in length and width by longitudinal and appositional growth, respectively, as described above. The common endocrine control of body composition will determine that linear growth drives bone mineral accrual. Indeed, as a result of bone development, BMC and BMD increase substantially during childhood as shown in published reference curves for DXA-derived bone mineral properties from 5 to 20 years of age [107]. Bone mineral acquisition follows different and specific age- and sex-patterns. Bone mass increases steadily during childhood, then its accretion accelerates after the onset of puberty and the adolescent growth spurt, peaking shortly after height peaks (Figure 9) [85].

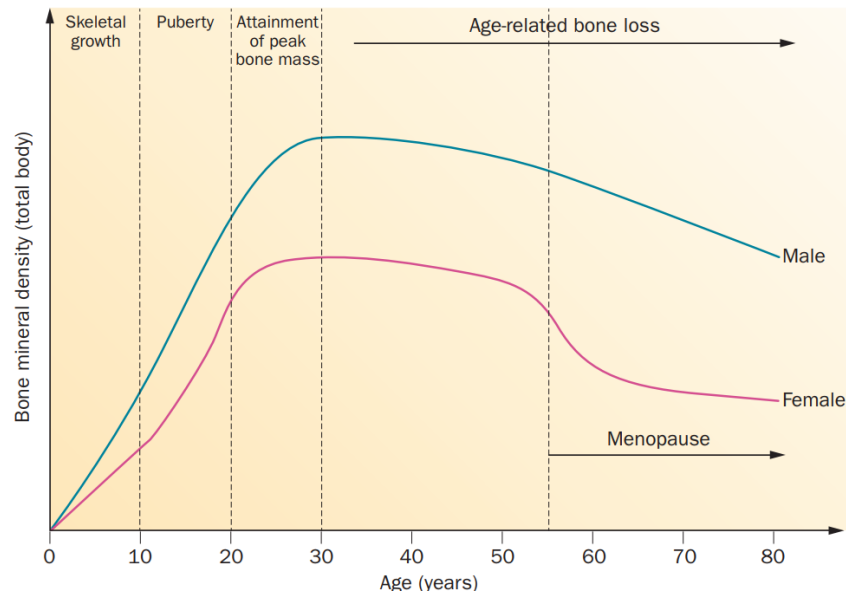


Figure 9. Overview of BMD changes throughout the life course (reproduced from Hendrickx *et al.*[123])

The most consistent framework to explain the action of environmental determinants on bone mineral accrual is the functional model of bone development, based on Frost's mechanostat theory, proposed by Rauch and Schoenau (Figure 10) [124]. This model builds on the premise that one-way relations of molecular, cellular and behavioral factors with bone accrual are overly simplistic, and bone development should not be seen as the result of the cumulative effect of the individual influences of these factors on bone mass or architecture. In fact, a self-assembly hypothesis may be correct for the process of biological skeletal patterning, occurring during the embryonic development but, once mineralization has started, it is more difficult to explain skeletal development on the basis of a similar self-assembly process [124]. Alternatively, and according to the mechanostat theory, rather than just being a passive recipient to external stimuli, bone tissue regulates itself with the aim of maintaining its structural integrity and strength. Accordingly, the central piece of bone regulation is the feedback loop between bone deformation (tissue strain) and bone strength. This means that bone physiology is a controlled effector-sensor system, where the purpose of homeostatic mechanisms is to maintain the strain (i.e. the mechanically induced deformation of bone) to a certain predetermined set point. When bone stability is challenged and bone deformation exceeds an acceptable level (the mechanostat set point), changes occur in order to add bone tissue at the location where it is mechanically necessary. This may be accomplished through changes in mass but also, and independently, through changes in the macro or microscopic architecture of bone tissue. During growth, bone accrual requires constant regulation due to the need for the skeletal framework

to adapt to changing external circumstances. At this period, changes in body size as a consequence of both an increase in bone length and an increase in muscle mass are seen as challenges to mechanical stability, and the resulting bone deformation is sensed by osteocytes, which lead to changes in bone mass and architecture to better adapt the skeleton to the load it bears [125]. Additionally, the mechanostat model assumes that, hormonal, nutritional or behavioral influences on bone homeostasis act by altering the mechanostat set point or the response to a disturbance to that point, i.e., by modifying the effect of mechanical strain on bone. Overall, the most relevant aspect of this model is that it places the maintenance of mechanical properties at the center of the bone development process [124]. It should be noted that, although mechanical stress from muscle contraction is already present during fetal development, leading to the beginning of bone mineralization in utero, dynamic strain-dependent bone signals that challenge mechanical stability become particularly relevant after birth.

The mechanostat theory, that places the bone functional requirement, in response to the mechanical stress exerted through gravitational and muscle forces, as the main osteogenic stimulus, has been very useful to explain the clear strong cross-sectional associations observed between current body size, both in weight and height, and bone mass and density in children [126]. At any given age, increased body size is associated with increased BMC and BMD reflecting, to a large extent, this adaptation of skeletal modeling to loading [117,127-134]. However, this model lacks a temporal dimension and may be limited to clarify potential long-term effects of changes in body size, such as those occurring during growth, on bone properties in a later period in life. Particularly, the long-term influence of increased overall exposure to body size, in dose and/or duration, on bone properties as well as whether there exist specific periods during growth, where the influence of changes in body size on bone properties are more likely to persist later in life, remain understudied.

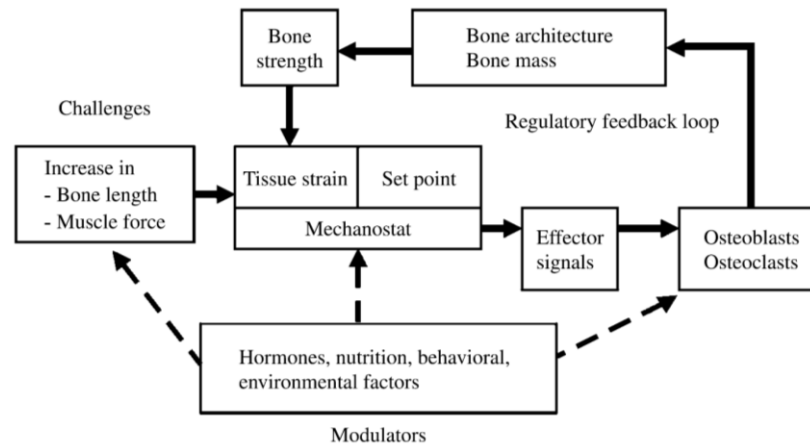


Figure 10. A functional model of bone development based on the mechanostat theory (reproduced from Rauch and Schoenau [124])

The increasing interest in modeling growth and identifying whether growth impacts on later health outcomes also led to the beginning of studies assessing associations between growth and bone health outcomes, including observations of the specific effect of early growth on pediatric bone physical properties [135-138].

A variety of methods are available to model longitudinal growth data and the choice of method will largely depend on the nature of the measurements available and whether assumptions inherent in the particular approach are met [138]. Most importantly, and particularly when the research objective is to detect the effects of growth in later health and disease outcomes, the selection of the statistical approach will largely depend on the question under consideration. While models of conditional growth, residual growth and multilevel linear spline, which provide measures of growth in different time intervals, allow for the exploration of whether particular sensitive periods exist for the effect of growth on a specific outcome, other group-based modeling methods, such as a growth mixture model, identify distinctive subgroups of individuals that follow similar patterns of growth, and allow relations between overall trajectories of growth and the outcome to be uncovered [138-141]. Differences in study results should be, in fact, understood in the context of the analytic strategies used.

To date, research on the effects of early growth during infancy and childhood on bone at a later time, has focused on examining the effect of specific growth periods on childhood bone mass and density, in order to identify sensitive periods. The results of these studies have suggested that the first three years of life are particularly relevant for the effect of growth velocity, both

height and weight velocity, on bone mineral accrual, with the magnitude of associations decreasing thereafter [135-137]. Regarding the sources of anthropometric measurements used to model growth, previous research has mainly relied on data obtained from large birth cohorts, where participants are followed at specified intervals, whose timing is defined *a priori* by researchers, and not necessarily related to the participants' ongoing health care utilization. Although anthropometric measurements collected in birth cohort follow-ups remain a valuable source of information to study growth, it may be possible to improve the detection of sensitive periods of growth if this data is complemented with anthropometric measurements obtained as part of routine health care. Routine health care registers represent an additional valuable source of information taking into account that visits to healthcare providers, when anthropometric measurements are obtained as part of routine health care, are taken at short time intervals. This was actually the starting point for paper II where weight and length/height growth trajectories, previously obtained through linear spline multilevel models on the basis of data collected during routine health examinations were used as exposures to identify sensitive periods for the effect of growth in the first 6 years of life on childhood bone mineralization.

Regarding the effect of overall trajectories of growth, less effort has been dedicated to assess the effect of differences in the overall shape of growth since birth on childhood bone mineral mass and density [138]. The hypothesis that different trajectories of growth in weight, particularly those that are related to an increased amount of exposure to body size, in dose and/or duration, may differently impact on later bone mineralization was the starting point for paper III.

An additional aspect that, so far, has not been analyzed is the impact of early life growth on childhood bone metabolism. Bone metabolism refers to the processes of bone resorption and bone formation that allows bone to adapt structure to function, in response to stress and other biomechanical forces. In line with the mechanostat theory, the bone functional requirement in response to mechanical stress bone metabolism will be responsible for adaptations of the processes of bone formation and resorption in such a way that final bone physical properties are maintained. Therefore, the study of bone metabolism during childhood may provide an important dynamic dimension that would not be captured by examining only the resulting physical properties [142,143]. During formation and resorption processes, bone metabolism markers are released into circulation. These markers can be measured in samples of blood and urine. In growing children, results from serum bone metabolism markers concentration are particularly challenging to interpret as their release into circulation reflect the whole set of

processes of bone remodeling, modeling and growth in length. As such, bone metabolism markers are mainly used in the clinical setting to diagnose and monitor pediatric diseases [143]. Consequently, it is not clear at present whether bone metabolism markers in children can be used as independent predictors of bone mass and density, particularly in what concerns generally healthy children from population-based samples. This previous observation, in addition to the current limited knowledge on relationships between trajectories of growth and bone formation and resorption was the starting point for paper IV.

1.8.3. Adiposity and childhood bone mass

Research on the relationship between fat mass and bone has been driven by the observation that increased weight was a protective factor for fragility fracture. The evidence to establish relationships between adipose tissue and the growing skeleton has been generated from two quite different sets of data that include epidemiological studies and laboratorial studies of physiological mechanisms.

At the population-level, research conducted on children has suggested that increased fat mass is a positive independent determinant of bone mass and size, similar to previous findings in adults [110,144-146]. As a consequence increased adiposity was considered protective against low bone strength in children. However, evidence over the last two decades demonstrating that obese children are over-represented in fracture groups [147,148] has raised concerns that excess fat mass could have a detrimental effect on bone development in children, by directly interfering with bone properties. Even though potential causative explanations for the increased risk of fracture in obese children includes greater propensity to falls or greater force generated on impact through falls [149,150], the hypothesis that excessive adipose tissue could have detrimental effects on bone mass led to a growing number of studies that assessed and suggested this deleterious effect [93,151,152].

Several potential mechanisms may explain direct and indirect effects from fat mass on skeletal development. Most of the positive effect of fat mass on bone mass is associated with mechanical loading that increases bone formation [153]. Not only does fat mass contribute to the direct mechanical action through increased gravitational loading, but also, increases in fat mass are associated with compensatory increases in muscle mass that promote bone formation [154].

In addition to the effects in increasing mechanical load, adipose tissue may influence bone metabolism through adipokines or other cytokines [155]. There is increasing evidence that the

hormone leptin, secreted by adipocytes, has a role in both the central and peripheral regulation of bone formation [156]. Through its effect as a growth factor on chondrocytes of skeletal growth centers, leptin directly exerts an osteogenic effect on bone. In addition, leptin has an indirect effect on bone formation by influencing other hormones affecting bone density (growth hormone, androgens, and cortisol). Leptin also seems to act as a negative regulator of bone formation via a central nervous system pathway [157]. It is also suggested that this adipokine may contribute to the radial and tibial microstructural change observed in obese children [158]. More recently, adiponectin was recognized as an important influence on bone mechanical properties that might have an even larger influence on bone phenotypes than leptin [159]. There is contradictory literature regarding adiponectin effects on bone from laboratorial studies but consistent inverse relationships between circulating adiponectin concentrations and BMD are described [160]. Additionally, adipose tissue produces inflammation-related cytokines, such as tumor necrosis factor α (TNF- α) and interleukin 6 (IL-6), which may have deleterious effects on bone [161]. Adipocytic estrogens are also likely to be involved in the relationships between fat and bone. Estrogen has a major role on the regulation of bone growth through its actions on osteoblasts, promoting accrual of bone mass on the cortical endosteal surface and in trabecular bone [162]. Alterations in the bone marrow compartment have also provided some insight into the mechanisms that may result in changes in bone density in childhood obesity [163,164]. Since visceral adiposity impairs growth hormone secretion and the response of growth hormone secretion to stimuli, this may have a negative impact on bone mass accrual [165].

Another way by which adiposity may affect bone mass is through its association with insulin resistance [166]. In prepubertal obese children, a lower BMC was observed in children with prediabetes than in those without prediabetes suggesting a negative effect of abnormal glucose regulation on the growing skeleton [167]. This may be explained by systemic inflammation associated with insulin resistance that activates bone resorption and further results in reduced BMD [168]. Other mechanisms for potential negative effects of impaired glucose metabolism on bone may be through alterations in calcium excretion or vitamin D metabolism, pituitary inhibition of growth hormone secretion or through increased concentrations of advanced glycation end products in collagen [165,167]. This pathway has gained major importance as a bone-pancreas endocrine loop has been recently postulated. There is a growing body of basic research demonstrating a tight reciprocal regulatory interaction between osteoblast and glucose metabolism, mediated to a large extent by insulin and osteocalcin. Insulin activates the osteoblast insulin receptor to increase bone formation and resorption, and releases osteocalcin

into the systemic circulation, which in turn can increase insulin sensitivity and pancreatic insulin secretion [169].

In view of all the potential mechanisms that contribute to explain the relationship between fat mass and bone properties in children, to date, the relationship between childhood obesity and bone mass remains controversial [145]. Conflicting results have been attributed to the possibility of changes in the relationship between fat and bone mass throughout different growth stages [144]. Additionally, it is possible that the detrimental impact of childhood obesity on bone mass in children may be observable only when fat mass accumulation reaches excessive levels [170,171]. Moreover, site-specific fat depots may also contribute to different results since it is believed that, in opposition to appendicular, visceral adiposity may be the one responsible for the negative impact of excessive fat mass on bone development, similar to what was described for associations with metabolic complications [170,172]. Furthermore, mechanical properties of bone were assessed through several methods, including DXA, peripheral quantitative computed tomography, and quantitative ultrasound, and studies have reported effects on some but not all measures of bone strength. There is also evidence that cortical or trabecular bone may be differentially affected by obesity. Trabecular bone is more likely to respond to the effects of fat mass as it is more available for metabolic processes [145].

Nevertheless, any mechanistic explanation of the effect of fat on bone should rely on valid effect estimates. There is a generalized observation of a crude positive association between adiposity and bone mass. However, results are much more heterogeneous when those associations are statistically corrected for overall body size or fat-free mass in order to overcome potential confounding. This issue deserves special attention since several studies have argued that “fat mass after adjustment for body weight” actually has a deleterious effect on bone [153,173-178]. Correction of variables such as fat mass for others to which they are closely related such as weight raises important validity concerns because of their close biological - and thus statistical - dependency [179]. Therefore, in order to test the potential for adiposity to specifically influence bone mass in children, the starting point for paper V was to use an alternative analytical approach to the inclusion of fat mass and body weight in the same regression analysis. We took advantage of data obtained outside the domain of physiology and used socioeconomic position as a benchmark, i.e. assuming that adiposity is socially-patterned, if adiposity is a major cause of bone mass we should also be able to detect a social pattern in bone mass. By quantifying the association between socioeconomic position and bone mass we inferred on the potential for a specific effect of fat on bone.

2. Study objectives

By using prospective data from the Generation XXI birth cohort our specific objectives, summarized in a schematic plan (Figure 11), were:

- 1.** To quantify the associations between gestational weight gain and bone mineralization in offspring, testing early pregnancy body mass index as an effect modifier (Paper I)
- 2.** To characterize the impact of early life growth on bone mineralization in childhood by:
 - a.** Identifying sensitive periods for the effect of early growth on childhood bone mass (Paper II)
 - b.** Assessing whether different trajectories of weight gain since birth influence childhood bone mineralization (Paper III)
- 3.** To describe markers of bone turnover in generally healthy children and assess their correlations with anthropometric growth and bone mineralization (Paper IV)
- 4.** To test the potential for adiposity to specifically influence bone mass in children, using common causes as a benchmark (Paper V)

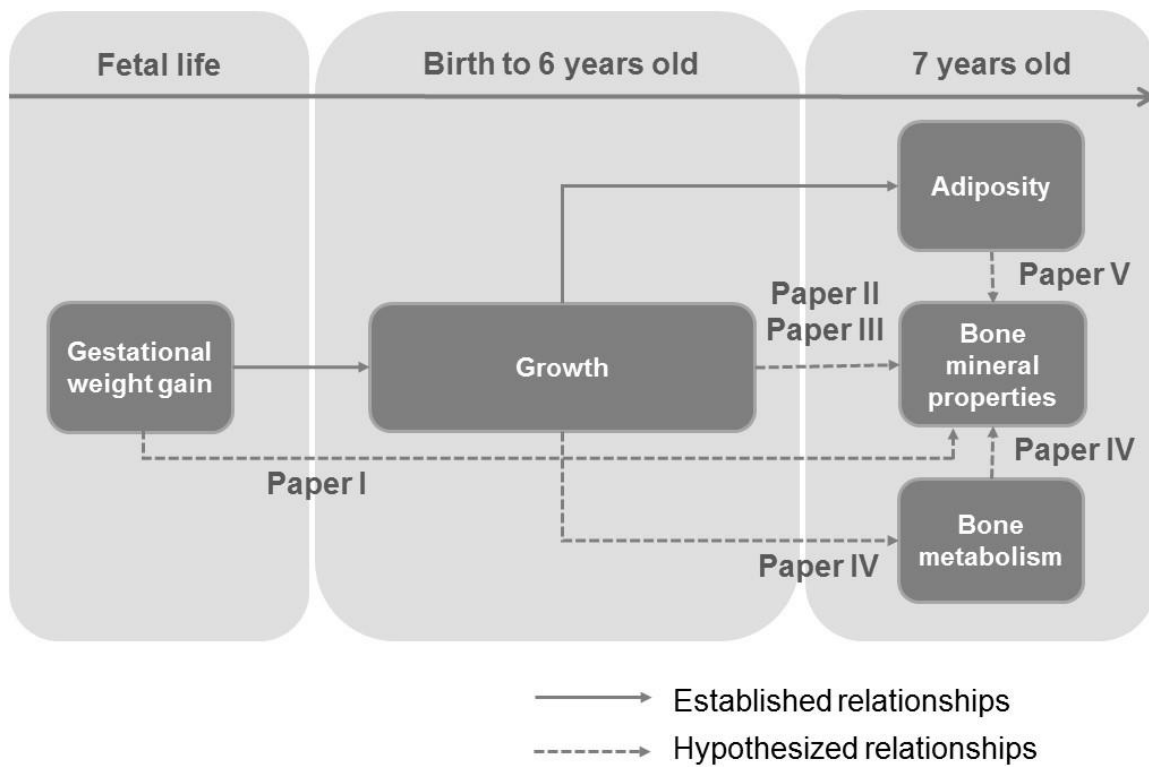


Figure 11. A conceptual framework of the effects of growth on childhood bone status

3. Participants and Methods

The objectives of this thesis were accomplished through the analysis of data obtained from the Generation XXI birth cohort study, from the baseline and follow-up evaluations at 4 and 7 years of age. Specifically, data from a consecutive subsample of 2408 children selected from the 7-years-old evaluation was used, for whom, besides the core variables common to the whole cohort, accepted to do an evaluation of musculoskeletal health and had a valid dual-energy X-ray absorptiometry (DXA) scan. A general description of Generation XXI participants and data collection procedures is provided below. Additional details about the study have been published elsewhere [180,181]. The selection of participants eligible for each paper depended on the specific objectives of the investigations and is described in detail in the methods sections of each individual chapter.

3.1. The Generation XXI cohort study

Generation XXI is the first Portuguese birth cohort study assembled during 2005 and 2006 in the Porto Metropolitan Region. It was established as a multi-purpose prospective population-based cohort that aims to characterize prenatal and postnatal growth and development, and identify their determinants, in order to better understand health in childhood and later in adolescence and adulthood, thereby contributing to health gains among the population.

3.1.1. Participants

Recruitment was conducted between April 2005 and August 2006 at the five public maternity units providing obstetrical and neonatal care covering, at the time, the metropolitan area of Porto, Portugal: *Centro Hospitalar de Vila Nova de Gaia*, *Centro Hospitalar do Porto - Maternidade de Júlio Dinis*, *Hospital de São João*, *Centro Hospitalar do Porto - Hospital de Santo António* and the *Unidade Local de Saúde de Matosinhos - Hospital Pedro Hispano*. All of these maternities corresponded to level III units, with differentiated perinatal support, and in 2004, were responsible for 91.6% of the deliveries in the whole catchment population, with the remaining occurring in private hospitals/clinics.

All women resident in the catchment area - one of the six municipalities of the metropolitan area of Porto (Figure 12) - and who delivered a live-born child with more than 23 weeks of gestation in one of the five units, during the cited period, were eligible to participate. Seventy percent of eligible mothers were invited and, of these, 91.4% accepted to participate. A total of 8647 infants and their mothers (n=8495) were enrolled in the cohort study.

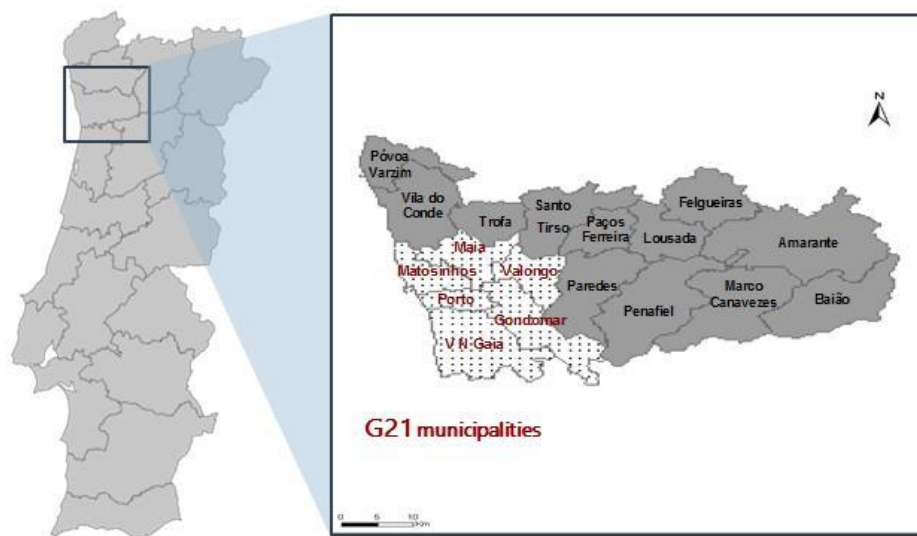


Figure 12. Representation of the six municipalities of the metropolitan area of Porto

3.1.2. Ethical considerations

The Generation XXI study protocol complies with the Ethical Principles expressed in the Declaration of Helsinki and was approved by the Ethics Committee of the *Hospital de São João* and the University of Porto Medical School. The study protocol also conforms with the national legislation and is registered with the Portuguese Data Protection Authority. Procedures were developed in order to guarantee data confidentiality and protection. All participants received an explanation on the purposes and design of the study. Written informed consent was obtained from all parents or legal guardians, and oral assent was obtained from children at each evaluation. Alternatively, verbal consent was explicitly solicited for the telephone interviews.

3.1.3. Data collection

At baseline, in the first 24 to 72 hours after delivery during the hospital stay, trained interviewers located at the five hospitals were responsible for presenting the Generation XXI study and inviting mothers. Data were collected in a face-to-face interview and clinical records at birth were reviewed by the trained interviewers.

Subsamples of the cohort were evaluated at the ages of 6 months, 15 months and 2 years ($n=1555$, $n=1043$ and $n=855$, respectively).

Four years after birth, between April 2009 and July 2011, a follow-up evaluation of the entire cohort was performed and 7459 children, corresponding to 86.3% of the cohort, were

reevaluated. Of these, 5987 children (69.2% of the cohort) were evaluated in a face-to-face interview, while for 1472 children (17.0% of the cohort), who were unable to attend an in-person evaluation, a shorter version of the questionnaire was completed by legal guardians in a telephone interview.

Between April 2012 and April 2014, all families were invited once again to attend the 7-year follow-up evaluation of the cohort. Overall, 6889 children were reassessed (79.7% of the entire cohort), of which 5849 children (67.6% of the cohort) were evaluated in-person and 1040 (12.0% of the cohort) provided information by telephone interview.

A subsequent evaluation of the cohort took place between July 2015 and July 2017, when children were 10 years of age, and 6397 children (76.0% of the cohort) were reassessed. Currently, the 13-year- old evaluation is ongoing; it started in August 2018 and it is expected to be completed in December 2019.

Follow-up evaluations of the cohort take place at the *Departamento de Ciências da Saúde Pública e Forenses, e Educação Médica* in the University of Porto Medical School. Standard procedures have been established and adopted in all evaluations. A multidisciplinary team of interviewers and health professionals, that is periodically trained and supervised by the Generation XXI administration team, is responsible for the application of structured questionnaires, performing the physical examination of children and biological parents and extracting information from clinical records and the National Health Service official health books.

The following description of procedures refers specifically to data collection sections for the baseline, age four and age seven follow-up evaluations as these were the periods of evaluation from which data to accomplish the objectives of this work were obtained.

a) Questionnaires

Face-to-face interviews using structured questionnaires designed by a multidisciplinary team were conducted to obtain information on the following areas:

- **Family demographic, socioeconomic, psychosocial circumstances and lifestyles:** including caregivers date of birth, educational level, working conditions and smoking, household income and crowding ,and other socioeconomic indicators of psychosocial adversity

- **Child and family medical history:** including existence of chronic diseases, experience of pain, intake of medication, vitamin or mineral supplements, healthcare use - including prenatal care - and maternal gynecologic and obstetric history
- **Maternal (during pregnancy) and child behaviors:** including food intake, sports and physical activity
- **Pre and throughout pregnancy anthropometrics:** at baseline, maternal weights at the beginning of pregnancy and immediately predelivery were self-reported by mothers

Data on sensitive topics such as household financial support needs, parental depressive symptoms and intimate personal behaviors (e.g. parental discipline) were gathered through self-administered questionnaires to parents or legal guardians and children, completed at the study site or at home.

b) Physical examination

Physical examination of the child included anthropometric, cardiovascular (including blood pressure assessments), respiratory and a dental evaluation. Measurement devices were carefully standardized and regularly calibrated.

- **Anthropometric assessment:** measurements of length/height and weight using infantometers or stadiometers and scales were conducted. Waist, hip, arm and leg circumferences were measured using flexible and non-distensible tapes, and body composition was assessed by bioelectric impedance (TANITA, Arlington Heights, Illinois, USA). All measurements were obtained while the child stood barefoot in light indoor clothing.
- **Forearm bone mineral density assessment:** assessment of forearm bone mineral density with a Lunar Peripheral Instant X-ray Imager (PIXI) device (GE Medical Systems, Madison, Wisconsin, USA) was conducted during the 7 years-old follow-up evaluation. The measurement was performed at the non-dominant distal forearm unless there was a reported fracture.

The physical examination of biological parents included anthropometric and blood pressure assessments.

c) Blood sample collection

In each evaluation, trained nurses collected child and maternal blood samples drawn from an antecubital vein after overnight fasting. All children were offered local dermal analgesia with lidocaine/prilocaine (EMLA®). Samples were centrifuged and sera were stored frozen at -80°C in the biobank at the University of Porto Medical School until analyses.

Determinations of bone metabolism markers

From the 5849 children evaluated in-person at 7 years of age, a subsample of 400 participants was selected to measure bone metabolism markers and bioactive molecules including serum 25-hydroxyvitamin D (25(OH)D), intact parathyroid hormone (PTH_i), calcium (Ca) and inorganic phosphorus (P_i).

The sample size was calculated based on expected correlations between bone parameters, in order to allow estimating a 0.15 linear correlation coefficient with an 85% power at a 0.05 significance level and selected based on the following characteristics: 1) to be a singleton full-term baby (more than 26 weeks of gestation); 2) to have a valid whole body DXA scan at 7 years (as described below), 3) to have a fasting blood sample collected in the morning with enough volume to perform the intended laboratory analyses and, although not exploited in the present work, 4) to have serum determinations of glucose and insulin from previous evaluations of the cohort and 5) to have information regarding maternal gestational diabetes.

All analytical measurements were conducted at the clinical pathology laboratory of the *Centro Hospitalar São João, EPE* in Porto and performed according the manufacturer's instructions regarding preventive maintenance, function checks, calibration, and quality control of both tests and equipment. Total alkaline phosphatase (tALP) was quantified by a spectrophotometric method. Osteocalcin (OC), β -crosslaps (β -CTx), and intact parathyroid hormone (PTH_i) measurements were performed by electro-chemiluminescence immunoassays (ECLIA). Serum 25-hydroxyvitamin D concentration (25(OH)D) was determined using a direct competitive chemiluminescence immunoassay. Calcium (Ca) was detected by a colorimetric assay and inorganic phosphorus (P_i) was determined by a colorimetric assay.

d) Review of clinical records, and the National Health Service official pregnancy and children's health book

At the baseline evaluation, obstetric clinical records held at the maternity units and the National Health Service official pregnancy booklet - a record of check-ups, ultrasounds, tests and medical notes provided as part of routine primary care to all pregnant women - were reviewed. These records were reviewed with the purpose of recovering only data that were missing from the baseline questionnaire, including data on prenatal care, pregnancy complications, pregnancy anthropometrics, and delivery and neonatal characteristics such as childbirth weight, length and gestational age.

At each follow-up evaluation, legal guardians were asked to bring their children's National Health Service official health and vaccination booklets so data on the child's development could be abstracted. Specifically, from the children's health book, which is a record of data obtained as part of children's routine health care, the research team abstracted all the length/height and weight measurements performed at every medical visit to a health professional.

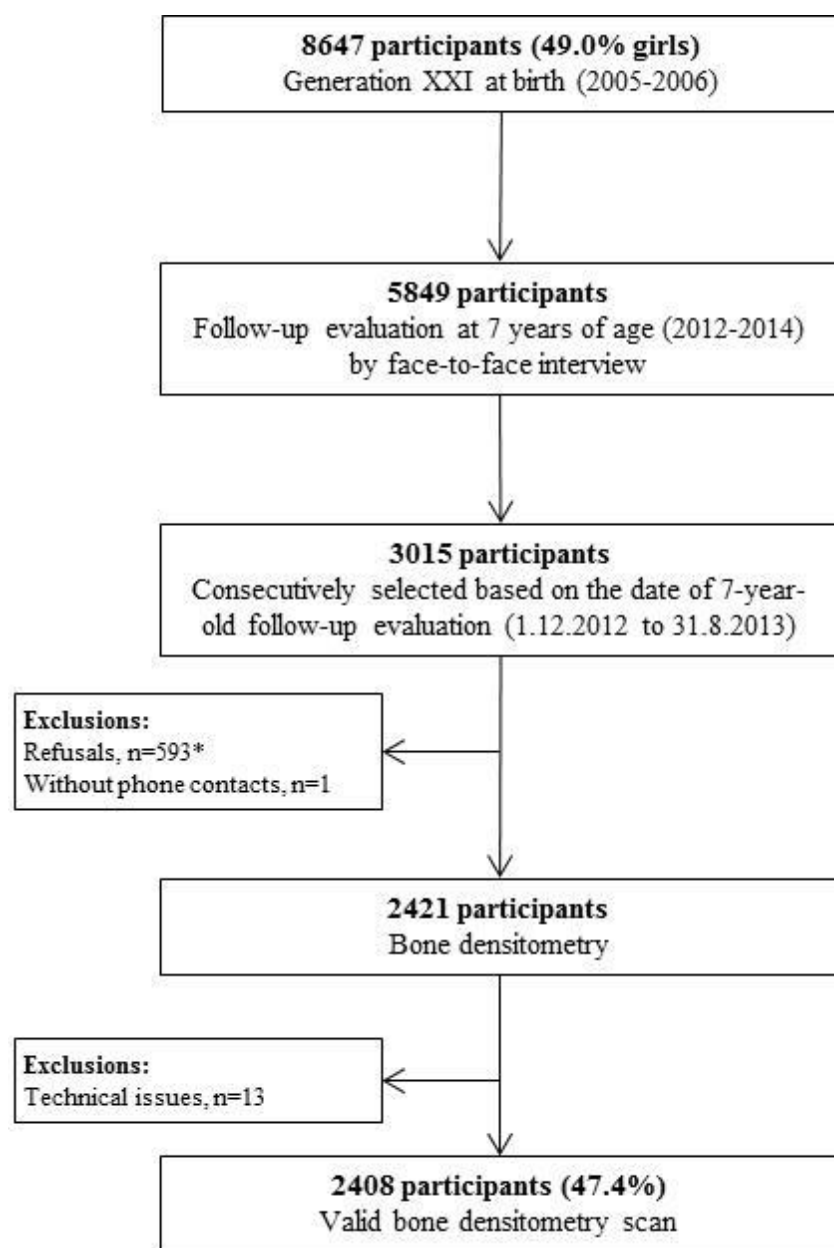
3.1.4. Additional wave of assessment for musculoskeletal evaluation

In the 7 years-old follow-up evaluation, a consecutive subsample of children who attended the face-to-face interviews between December 1, 2012 and August 31, 2013 was invited to an additional wave to assess bone mineral properties and sagittal posture. The selection of this subsample was based solely on the funding schedule and equipment availability (Figure 13). According to standard manufacturer's protocol, whole body DXA scans were performed using a Hologic Discovery QDR 4500W device (Hologic Inc., Bedford, Massachusetts, USA), software version 13.3.0.1., while children were barefoot in light indoor clothing and without metal accessories. Nine trained radiology technicians were involved in the DXA evaluations. To ensure that the lines between adjacent subregions of the body were placed correctly and to exclude images showing unacceptable artefacts, two trained operators checked each scan in two independent moments, immediately after acquisition and in a later validation. Standard quality assurance tests were performed daily using the spine phantom according to the manufacturer's instructions and the coefficient of variation obtained from repeated phantom measurements was below 1%. Bone mineral content (BMC) was obtained and areal bone mineral density (aBMD) was expressed as BMC (in g) per projected bone area (in cm²). A measure of size-corrected BMC (scBMC) was derived separately for girls and boys by linear regression of BMC on bone area and the addition of the resulting residuals to the mean

sample's BMC [182]. Body composition measures, including fat mass (kg) and lean mass (kg), were also obtained during DXA scanning. As recommended by the International Society for Clinical Densitometry for research in pediatric populations, total body less head (subtotal) measures were used [61].

In this evaluation weight was measured once again in using a digital scale (Xinyu Electronic Co., Limited, Zhongshan, China) and height was measured using a wall stadiometer (SECA, Hamburg, Germany).

Of the 3015 children who were eligible to attend the musculoskeletal evaluation (corresponding to 43.8% of the participants in the 7 years follow-up and 34.8% of the entire cohort), 80.3% (n=2421) agreed to participate and attended the scheduled assessment at *Escola Superior De Tecnologia da Saúde do Instituto Politécnico do Porto*. DXA scans were conducted successfully in 2408 children (79.9% of the eligible sample and 27.8% of the entire cohort). This additional wave of assessment took place between March 2013 and February 2014 in a median (P25, P75) of 62 (15, 225) days after the 7 years-old evaluation (Figure 13).



* Fourth box - based on the date of the 7-y participants who refused to participate in the musculoskeletal evaluation (n=257) + participants who scheduled three different appointments but did not show up for evaluation, or did not respond to our invitation after at least five attempts (n=335) + participant with cerebral palsy that did not cooperate in the evaluation (n=1)

Figure 13. Flow-chart of participants evaluated with a valid dual-energy X-ray absorptiometry scan

Figure 14 presents the outline of each of the studies conducted regarding main exposures and sample size.

PAPER	I	II	III	IV	V
MAIN EXPOSURES	Maternal gestational weight gain	Age specific growth velocities	Trajectories of growth	Bone metabolism markers	Fat mass
SAMPLE SIZE	2167	1853	1889	395	2408

Figure 14. Schematic presentation of the main exposures and sample size used in Papers I-V

Integration of the subsets of Generation XXI participants with DXA scan at 7 years of age (n=2408), included in the growth analysis (n=5282) and with information regarding maternal gestational weight gain and prepregnancy body mass index (n=8033) are represented in Figure 15. Intentionally, the subset of participants with bone metabolism markers (n=395) is not represented in this scheme as it was sampled from the participants with DXA scan.

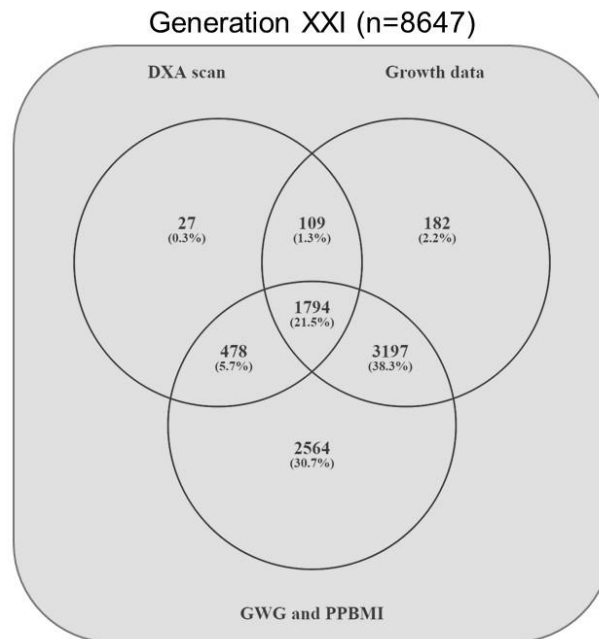


Figure 15. Subsets of participants within the Generation XXI cohort with information for the main variables of interest. The numbers shown in each subset do not match exactly the final numbers of participants analyzed in each article due to specific exclusions related to missing data for confounders or covariates. DXA - dual-energy X-ray absorptiometry; GWG – Gestational weight gain; PP BMI - Prepregnancy body mass index

4. Results

4.1. Description of bone mineral properties in the Generation XXI birth cohort

In the Generation XXI study, 2408 participants corresponding to 1142 girls (27% of all the girls in the cohort) and 1266 boys (28.7% of all the girls in the cohort), had data on DXA-derived bone measures at 7 years of age. Mean (standard-deviation) subtotal bone mineral content (BMC) and areal density (aBMD) were higher in boys [BMC: 600.9 (85.6) g and aBMD: 0.624 (0.053) g/cm²] than in girls [BMC: 591.8 (85.6) g and aBMD: 0.624 (0.053) g/cm²], p-values of 0.009 and <0.001 (Figure 16). As expected, a clear increasing trend in bone mineral properties with increasing age was observed [BMC: Pearson correlation coefficient (r)=0.33, p<0.001; aBMD: r=0.29, p<0.001] (Figure 17).

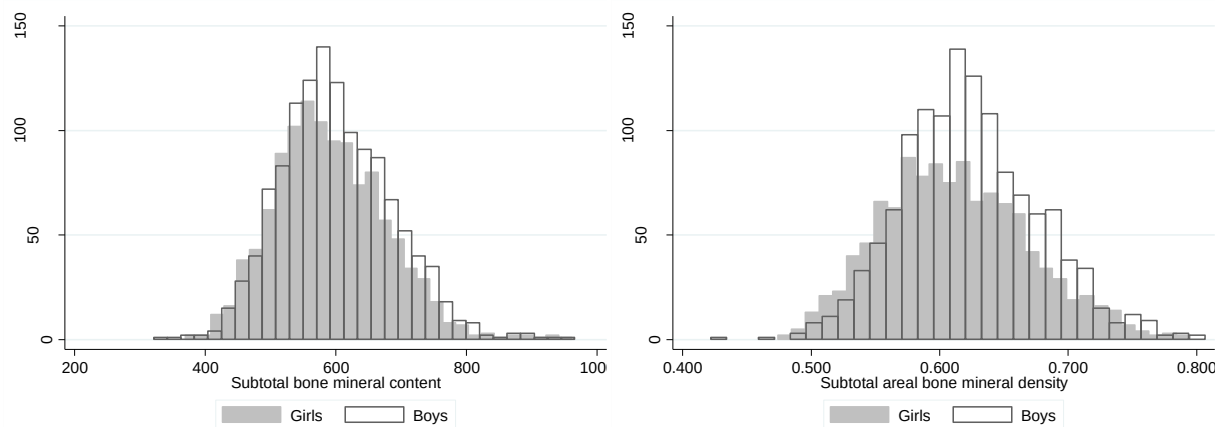


Figure 16. Distribution of subtotal bone mineral properties by sex

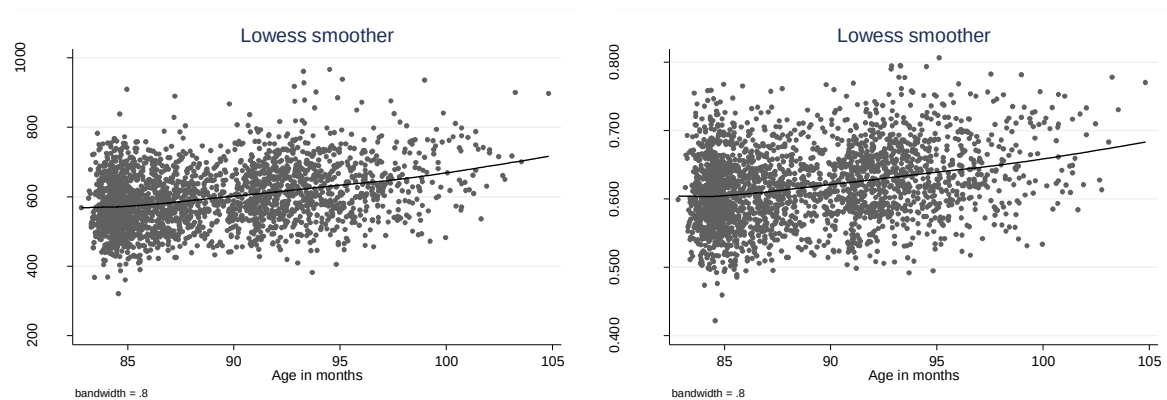


Figure 17. Relationship between age and subtotal bone mineral properties

Age- and sex-specific z-scores were computed for BMC and aBMD based on the method, and reference values from the Bone Mineral Density in Childhood Study, published by Zemel *et al* [107,183]. In comparison to the non-black children from the United States reference population, children from the Generation XXI had a slightly lower mean BMC and aBMD [mean (SD) z-scores: girls: -0.05 (0.92) and boys: -0.02 (0.86)].

Tables 1 to 7 present the mean values of BMC and aBMD in girls and boys, according to sociodemographic, anthropometric and behavioral characteristics. Mean BMC and aBMD increased linearly with height, body weight as well as with body mass index. Positive crude dose-response associations between BMC and aBMD and all measures of body composition (subtotal lean and fat mass, and body mass percentage), both in girls and in boys, were found (Table 1).

Table 1. Mean (SD) bone mineral content (g) and areal density (g/cm²) at 7 years of age, in girls and boys, according to current anthropometric characteristics

		Girls			Boys		
		n (%)	BMC	aBMD	n (%)	BMC	aBMD
Height z-score ^a							
	< -2.00	10 (0.9)	461.3 (44.5)	0.535 (0.033)	10 (0.8)	412.2 (31.8)	0.522 (0.026)
	-2.00 to -1.1	92 (8.1)	487.7 (45.9)	0.550 (0.036)	103 (8.1)	503.1 (50.0)	0.569 (0.037)
	-1.0 to 0.1	369 (32.3)	544.4 (55.8)	0.583 (0.042)	403 (31.8)	556.7 (57.0)	0.599 (0.041)
	0.0 to 0.9	457 (40.0)	610.5 (63.2)	0.624 (0.046)	471 (37.2)	616.1 (65.4)	0.632 (0.044)
	1 to 1.9	180 (15.8)	674.0 (66.9)	0.660 (0.046)	240 (19.0)	670.2 (67.5)	0.661 (0.043)
	≥ 2	34 (3.0)	739.6 (89.4)	0.702 (0.047)	39 (3.1)	756.2 (82.5)	0.711 (0.044)
	p (trend)		<0.001	<0.001		<0.001	<0.001
Weight z-score ^a							
	< -2.00	3 (0.3)	445.9 (42.0)	0.519 (0.026)	8 (0.6)	418.2 (36.2)	0.516 (0.033)
	-2.00 to -1.1	50 (4.4)	468.2 (46.4)	0.530 (0.031)	56 (4.4)	471.2 (43.1)	0.544 (0.031)
	-1.0 to 0.1	228 (20.0)	521.0 (51.1)	0.563 (0.033)	274 (21.6)	540.0 (50.4)	0.584 (0.033)
	0.0 to 0.9	393 (34.4)	579.0 (58.6)	0.597 (0.035)	435 (34.4)	591.0 (60.1)	0.615 (0.036)
	1 to 1.9	284 (24.9)	630.0 (61.1)	0.638 (0.035)	301 (23.8)	644.0 (69.5)	0.646 (0.037)
	≥ 2	184 (16.1)	683.8 (82.6)	0.688 (0.043)	192 (15.2)	688.3 (77.4)	0.691 (0.041)
	p (trend)		<0.001	<0.001		<0.001	<0.001

BMI categories ^b

Underweight	2 (0.2)	525.6 (51.8)	0.562 (0.020)	4 (0.3)	419.9 (34.8)	0.510 (0.026)
Normal weight	673 (58.9)	560.3 (72.8)	0.585 (0.044)	782 (61.8)	576.5 (76.3)	0.604 (0.044)
Overweight	271 (23.7)	620.5 (78.6)	0.632 (0.046)	272 (21.5)	624.8 (78.2)	0.639 (0.044)
Obese	196 (17.2)	661.0 (81.2)	0.678 (0.045)	208 (16.4)	665.2 (83.2)	0.679 (0.048)
p (trend)		<0.001	<0.001		<0.001	<0.001

Quarter of subtotal fat mass (kg)

< 5.3	181 (15.8)	510.1 (60.6)	0.558 (0.039)	448 (35.4)	552.1 (72.3)	0.593 (0.044)
5.3 to 6.8	280 (24.5)	561.0 (61.4)	0.585 (0.037)	312 (24.6)	593.8 (69.7)	0.615 (0.041)
6.9 to 9.3	320 (28.0)	592.8 (67.7)	0.608 (0.041)	276 (21.8)	632.3 (70.8)	0.638(0.042)
≥ 9.4	361 (31.6)	655.8 (78.8)	0.664 (0.047)	230 (18.2)	668.1 (84.6)	0.677 (0.049)
p (trend)		<0.001	<0.001		<0.001	<0.001

Quarter of subtotal lean mass (kg)

< 13.2	422 (37.0)	519.1 (49.4)	0.563 (0.033)	207 (16.4)	499.4 (49.5)	0.559 (0.033)
13.2 to 14.5	280 (24.5)	586.8 (47.9)	0.607 (0.031)	320 (25.3)	559.2 (42.3)	0.597 (0.025)
14.6 to 16.1	247 (21.6)	636.6 (52.0)	0.641 (0.030)	337 (16.6)	605.0 (50.8)	0.626 (0.029)
≥ 16.2	193 (16.9)	700.7 (72.3)	0.691 (0.039)	402 (31.8)	683.0 (69.1)	0.676 (0.041)
p (trend)		<0.001	<0.001		<0.001	<0.001

Quarter of body fat mass percentage (%)

< 25.9	120 (10.5)	540.8 (74.9)	0.577 (0.045)	488 (38.6)	575.7 (78.3)	0.607 (0.046)
25.9 to 30.5	256 (22.4)	562.3 (74.1)	0.586 (0.044)	355 (28.0)	594.8 (85.0)	0.616 (0.051)
30.6 to 35.9	352 (30.8)	584.2 (78.2)	0.601 (0.049)	240 (19.0)	623.2 (76.1)	0.635 (0.046)
≥ 36.0	414 (36.2)	631.2 (84.5)	0.647 (0.055)	183 (14.4)	651.0 (88.6)	0.668 (0.056)
p (trend)		<0.001	<0.001		<0.001	<0.001

^a World Health Organization reference criteria. Age- and sex-specific weight and height z-scores established according to the World Health Organization [184].

^b According to the World Health Organization classification for BMI-for-age z-score: underweight [<-2 standard deviations (SD)]; Normal weight (≥-2 SD and $\leq+1$ SD); Overweight ($>+1$ SD and $\leq+2$ SD); Obese ($>+2$ SD) [184,185].

A decreasing trend of the child's BMC and aBMD was found with increasing educational level of main and secondary caregivers. In boys, an increasing household income was negatively related with aBMD only. No other differences in mean BMC and aBMD according to employment status of the main or the secondary caregiver, household income, family home ownership, household crowding, family concern about household expenses, the child's type of school, main caregiver marital status or maternal country of birth, were found (Table 2).

Table 2. Mean (SD) bone mineral content (g) and areal density (g/cm²) at 7 years of age, in girls and boys, according to socioeconomic characteristics at the 7 years of age follow-up evaluation

		Girls			Boys		
		n (%)	BMC	aBMD	n (%)	BMC	aBMD
Main caregiver educational level ^a (years of schooling)							
	≤ 9	434 (38.0)	597.2 (89.4)	0.618 (0.060)	467 (36.9)	605.1 (83.1)	0.627 (0.053)
	10 to 12	347 (30.4)	596.4 (80.6)	0.614 (0.054)	402 (31.8)	603.6 (89.3)	0.626 (0.054)
	> 12	359 (31.4)	580.8 (84.9)	0.603 (0.055)	394 (31.1)	594.1 (83.9)	0.618 (0.051)
	Missing	2 (0.2)	578.7 (41.7)	0.604 (0.038)	3 (0.2)	497.6 (82.9)	0.566 (0.040)
	p (trend)		0.006	0.001		0.047	0.018
Secondary caregiver educational level ^b (years of schooling)							
	≤ 9	556 (49.5)	597.2 (86.7)	0.617 (0.059)	575 (46.2)	604.0 (81.8)	0.626 (0.052)
	10 to 12	300 (26.7)	588.6 (85.4)	0.609 (0.056)	365 (29.3)	600.6 (89.2)	0.624 (0.055)
	> 12	252 (22.4)	581.7 (83.9)	0.603 (0.053)	284 (22.8)	593.0 (88.7)	0.616 (0.052)
	Missing	16 (1.4)	623.6 (75.9)	0.632 (0.053)	20 (1.6)	601.6 (76.5)	0.627 (0.053)
	p (trend)		0.009	0.001		0.020	0.008
Main caregiver employment status ^a							
	Employed	888 (77.8)	594.0 (85.2)	0.613 (0.056)	982 (77.6)	603.7 (87.1)	0.625 (0.054)
	Unemployed	202 (17.7)	585.4 (84.5)	0.609 (0.058)	216 (17.1)	591.1 (79.4)	0.618 (0.051)
	Stay-at-home	37 (3.2)	567.7 (90.8)	0.598 (0.063)	44 (3.5)	593.4 (74.6)	0.614 (0.048)
	Other	14 (1.2)	613.0 (104.4)	0.628 (0.066)	23 (1.8)	591.6 (90.8)	0.621 (0.064)
	Missing	1 (0.1)	549.2	0.577	1 (0.1)	531.8	0.588
	p (ANOVA)		0.139	0.243		0.209	0.148

**Secondary caregiver
employment status ^b**

Employed	939 (83.5)	592.0 (85.5)	0.612 (0.057)	1044 (83.9)	601.3 (86.8)	0.623 (0.054)
Unemployed	113 (10.0)	590.1 (83.9)	0.612 (0.058)	127 (10.2)	597.0 (73.2)	0.624 (0.047)
Stay-at-home	14 (1.2)	612.0 (126.8)	0.614 (0.079)	17 (1.4)	597.1 (101.8)	0.622 (0.071)
Other	54 (4.8)	590.4 (84.3)	0.612 (0.061)	53 (4.3)	594.5 (85.8)	0.621 (0.055)
Missing	4 (0.4)	552.3 (86.1)	0.586 (0.047)	3 (0.2)	569.1 (33.6)	0.601 (0.022)
p (ANOVA)		0.149	0.264		0.072	0.076

**Household income
(EUR/month)**

≤ 1000	302 (26.4)	596.7 (90.0)	0.616 (0.061)	332 (26.2)	603.8 (84.5)	0.627 (0.055)
1001 to 2500	675 (59.1)	591.2 (83.8)	0.612 (0.056)	733 (57.9)	599.0 (83.8)	0.623 (0.052)
> 2500	141 (12.4)	579.5 (81.7)	0.602 (0.053)	180 (14.2)	603.4 (94.0)	0.622 (0.056)
Not known or Undisclosed	23 (2.0)	621.8 (96.8)	0.632 (0.060)	21 (1.7)	601.2 (91.3)	0.626 (0.058)
Missing	1 (0.1)	549.2	0.577	0	NA	NA
p (trend)		0.082	0.034		0.715	0.297

Family home ownership

No	857 (75.0)	590.7 (84.0)	0.611 (0.056)	966 (76.30)	599.5 (86.4)	0.623 (0.053)
Yes	271 (23.7)	595.3 (91.1)	0.615 (0.060)	289 (22.83)	605.2 (83.5)	0.627 (0.054)
Missing	14 (1.2)	589.9 (76.2)	0.613 (0.057)	11 (0.87)	619.6 (65.3)	0.629 (0.044)
p (t test)		0.450	0.394		0.319	0.269

**Household crowding index
(persons /room)**

< 1.0	242 (21.2)	595.6 (78.2)	0.614 (0.053)	293 (23.1)	604.6 (86.4)	0.626 (0.052)
1.0	452 (39.6)	591.8 (87.5)	0.611 (0.058)	507 (40.0)	599.1 (85.8)	0.623 (0.052)
1.1-2	392 (34.3)	592.8 (88.1)	0.614 (0.058)	399 (31.5)	600.9 (84.0)	0.624 (0.054)
> 2	31 (2.7)	554.6 (71.6)	0.589 (0.049)	40 (3.2)	593.8 (101.0)	0.617 (0.061)
Missing	25 (2.2)	586.0 (90.4)	0.604 (0.061)	27 (2.1)	606.9 (71.4)	0.624 (0.050)
p (trend)		0.226	0.571		0.400	0.232

**Family concern about
household expenses**

Never/rarely	65 (5.7)	592.4 (79.8)	0.609 (0.050)	83 (6.6)	611.9 (87.3)	0.632 (0.054)
Sometimes/a lot of times	269 (23.6)	584.0 (92.7)	0.606 (0.062)	303 (23.9)	599.5 (87.5)	0.632 (0.054)
Almost always/always	800 (70.1)	594.0 (83.1)	0.614 (0.056)	874 (69.0)	600.4 (84.8)	0.623 (0.053)
Missing	8 (0.7)	629.7 (117.9)	0.636 (0.067)	6 (0.5)	602.8 (91.5)	0.633 (0.070)
p (trend)		0.208	0.070		0.624	0.514

Child's type of school

Public	1019 (89.2)	591.5 (85.4)	0.612 (0.057)	1135 (89.6)	600.9 (84.8)	0.624 (0.053)
Private	114 (10.0)	595.4 (87.5)	0.610 (0.055)	120 (9.5)	606.0 (92.1)	0.625 (0.052)
Other	7 (0.6)	590.8 (110.9)	0.601 (0.060)	10 (0.8)	543.0 (84.2)	0.601 (0.052)
Missing	2 (0.2)	550.0 (1.1)	0.578 (0.001)	1 (0.1)	653.0	0.674
p (t-test)		0.641	0.676		0.527	0.845

Main caregiver marital status

Married/committed	1012 (88.62)	590.7 (86.3)	0.612 (0.058)	1114 (87.99)	600.7 (85.4)	0.623 (0.053)
Single	129 (11.30)	600.7 (80.0)	0.618 (0.052)	151 (11.93)	603.0 (87.0)	0.626 (0.058)
Missing	1 (0.09)	549.2	0.577	1 (0.08)	575.5	0.593
p (t-test)		0.212	0.263		0.759	0.598

Maternal country of birth

Portuguese	1044 (91.42)	592.7 (86.5)	0.613 (0.058)	1162 (91.79)	601.5 (86.2)	0.624 (0.053)
Non-Portuguese European	8 (0.70)	601.1 (45.2)	0.619 (0.039)	15 (1.18)	590.3 (84.3)	0.615 (0.051)
African	6 (0.53)	631.4 (67.5)	0.632 (0.027)	9 (0.71)	595.3 (54.6)	0.623 (0.033)
South American	21 (1.84)	606.6 (71.7)	0.632 (0.027)	13 (1.03)	606.4 (91.0)	0.634 (0.061)
Other	1 (0.09)	517.7	0.562	0		
Missing	62 (5.43)	567.4 (76.4)	0.599 (0.052)	67 (5.29)	593.8 (77.4)	0.617 (0.049)
p (ANOVA)		0.141	0.112		0.470	0.375

^a 97.9% biological mother; 1.4% biological father; 0.7% other

^b 87.2% biological father; 5.5% grandmother; 2.0% non-biological father; 0.8% biological mother; 4.5% other.

Excluding participants without a second caregiver (n=18 girls and 22 boys).

NA, not applicable

Boys born to women who smoked during pregnancy or who developed hypertensive disorders of pregnancy had increased bone mineral properties at 7 years. We found a trend for lower bone mineral properties at 7 years of age in children born from a multiple pregnancy, in comparison with singletons, however it was not consistent for both bone mineral properties (Table 3).

Table 3. Mean (SD) bone mineral content (g) and areal density (g/cm²) at 7 years of age, in girls and boys, according to maternal behaviors, clinical conditions and health care use during pregnancy

		Girls			Boys		
		n (%)	BMC	aBMD	n (%)	BMC	aBMD
Smoking status during pregnancy							
	No	895 (78.4)	592.7 (86.7)	0.612 (0.057)	1002 (79.2)	598.6 (83.5)	0.621 (0.052)
	Yes	239 (20.9)	587.4 (80.8)	0.611 (0.058)	252 (19.9)	606.3 (90.6)	0.630 (0.057)
	Missing	8 (0.7)	623.8 (100.8)	0.631 (0.064)	12 (1.0)	684.0 (107.0)	0.669 (0.062)
	p (t test)		0.394	0.664		0.195	0.017
Alcohol drinking during pregnancy							
	No	922 (80.7)	591.2 (86.3)	0.612 (0.057)	1050 (82.9)	600.2 (86.5)	0.623 (0.054)
	Yes	142 (12.4)	590.0 (81.6)	0.609 (0.054)	154 (12.2)	602.4 (81.3)	0.625 (0.051)
	Missing	78 (6.8)	602.0 (85.4)	0.620 (0.059)	62 (4.9)	609.7 (80.2)	0.624 (0.050)
	p (t test)		0.871	0.594		0.768	0.650
Number of fetuses							
	Singleton	1088 (95.3)	593.1 (86.0)	0.613 (0.057)	1209 (95.5)	601.7 (86.0)	0.624 (0.053)
	Multiple	54 (4.7)	565.0 (72.0)	0.600 (0.052)	57 (4.5)	583.8 (73.0)	0.610 (0.046)
	p (t test)		0.018	0.103		0.121	0.054
Gestational diabetes							
	No	1086 (95.1)	591.6 (85.7)	0.612 (0.057)	1178 (93.0)	600.4 (85.7)	0.623 (0.053)
	Yes	53 (4.6)	601.8 (81.3)	0.620 (0.056)	82 (6.5)	606.5 (82.3)	0.631 (0.051)
	Missing	3 (0.3)	500.5 (82.0)	0.544 (0.036)	6 (0.5)	635.2 (109.4)	0.651 (0.066)
	p (t test)		0.397	0.320		0.529	0.201
Hypertensive disorders of pregnancy							
	No	1116 (97.7)	591.7 (85.3)	0.612 (0.057)	1234 (97.5)	599.3 (84.5)	0.622 (0.053)
	Yes	23 (2.1)	607.7 (97.1)	0.625 (0.061)	26 (2.0)	669.3 (101.7)	0.668 (0.058)
	Missing	3 (0.3)	500.5 (82.0)	0.544 (0.036)	6 (0.5)	635.2 (109.4)	0.651 (0.066)
	p (t test)		0.376	0.275		<0.001	<0.001

First antenatal visit**(weeks of gestation)**

≤ 12	989 (86.6)	590.5 (87.3)	0.611 (0.058)	1118 (88.3)	601.2 (85.9)	0.624 (0.053)
> 12	81 (7.1)	601.6 (73.8)	0.617(0.051)	82 (6.5)	596.0 (84.5)	0.622 (0.051)
Missing	72 (6.3)	599.9 (73.6)	0.617 (0.052)	66 (5.2)	602.6 (81.2)	0.622 (0.051)
p (t test)		0.267	0.407		0.600	0.791

A trend for increased bone mineral properties at 7 years was observed in girls and boys born with great weight for gestational age. There were no clear associations between BMC and aBMD at 7 years old and type of delivery, gestational age at birth, levels of cord blood adipokines, growth during the immediate postnatal period (newborn weight change) or duration of exclusively breastfeeding (Table 4).

Table 4. Mean (SD) bone mineral content (g) and areal density (g/cm²) at 7 years of age, in girls and boys, according to birth and infant characteristics

		Girls			Boys		
		n (%)	BMC	aBMD	n (%)	BMC	aBMD
Type of delivery							
	Vaginal	710 (62.2)	590.4 (83.7)	0.610 (0.056)	750 (59.2)	601.7 (85.4)	0.624 (0.053)
	Caesarean section	432 (37.8)	594.2 (88.6)	0.615 (0.058)	516 (40.8)	599.8 (85.8)	0.623 (0.053)
	p (t test)		0.463	0.152		0.704	0.791
Gestational age at birth							
(weeks)							
	< 37 (preterm)	97 (8.5)	580.7 (92.9)	0.608 (0.059)	112 (8.8)	592.6 (84.6)	0.617 (0.055)
	≥ 37 (full term)	1045 (91.5)	592.8 (84.9)	0.613 (0.057)	1153 (91.1)	601.8 (85.7)	0.624 (0.053)
	Missing	0			1 (0.1)	570.5	0.626
	p (t test)		0.184	0.414		0.279	0.164
Weight for gestational age ^a							
	Small	171 (15.0)	566.1 (83.3)	0.599 (0.058)	181 (14.3)	578.4 (82.8)	0.613 (0.053)
	Adequate	867 (75.9)	595.1 (84.2)	0.613 (0.056)	992 (78.4)	604.4 (85.0)	0.625 (0.053)
	Large	50 (4.4)	652.2 (93.4)	0.651 (0.062)	35 (2.8)	648.4 (102.7)	0.649 (0.055)
	Missing	54 (4.7)	565.0 (72.0)	0.600 (0.052)	58 (4.6)	583.6 (72.4)	0.611 (0.046)
	p (trend)		<0.001	<0.001		<0.001	<0.001
Quarters of umbilical cord							
blood adiponectin (µg/ml)							
	< 24.7	51 (19.0)	584.1 (85.2)	0.612 (0.059)	90 (30.8)	595.6 (83.7)	0.617 (0.052)
	24.7 to 31.3	82 (30.5)	576.9 (80.8)	0.604 (0.056)	59 (20.2)	570.3 (78.3)	0.607 (0.051)
	31.4 to 38.2	71 (26.4)	588.5 (79.0)	0.609 (0.058)	72 (24.7)	583.2 (84.6)	0.614 (0.054)
	≥ 38.3	65 (24.2)	572.1 (78.0)	0.597 (0.055)	71 (24.3)	586.3 (81.1)	0.617 (0.049)
	Not measured	873	595.4 (86.9)	0.614 (0.057)	974	605.7 (86.0)	0.626 (0.053)
	p (trend)		0.729	0.256		0.381	0.848

Quarters of umbilical cord

blood leptin (ng/ml)

< 9.5	45 (16.7)	580.0 (78.9)	0.606 (0.051)	98 (33.6)	592.4 (90.2)	0.615 (0.055)
9.5 to 14.2	52 (19.3)	557.3 (61.2)	0.589 (0.045)	86 (29.4)	578.5 (79.1)	0.610 (0.052)
14.3 to 23.8	69 (25.6)	589.6 (87.1)	0.610 (0.062)	71 (24.3)	593.1 (75.0)	0.621 (0.049)
> 23.9	103 (38.3)	585.5 (83.6)	0.609 (0.060)	37(12.7)	566.6 (80.0)	0.607 (0.048)
Not measured	873	595.4 (86.9)	0.614 (0.057)	974	605.7 (86.0)	0.626 (0.053)
p (trend)		0.424	0.552		0.245	0.675

Quarters of umbilical cord

blood resistin (ng/ml)

< 47.5	54 (20.1)	578.9 (76.8)	0.606 (0.057)	87 (29.8)	597.2 (82.3)	0.620 (0.052)
47.5 to 60.8	65 (24.2)	577.3 (76.2)	0.602 (0.056)	75 (25.7)	565.8 (78.2)	0.603 (0.052)
60.9 to 79.5	71 (26.4)	583.8 (88.2)	0.608 (0.061)	70 (24.0)	595.1 (93.6)	0.622 (0.056)
≥ 79.6	79 (29.4)	580.1 (80.0)	0.603 (0.054)	60 (20.6)	580.3 (69.1)	0.611 (0.042)
Not measured	873	595.4 (86.9)	0.614 (0.057)	974	605.7 (86.0)	0.626 (0.053)
p (trend)		0.886	0.909		0.432	0.636

Quarters of newborn weight

change (%)

< -8.0	78 (27.4)	571.3 (85.7)	0.598 (0.059)	78 (24.7)	595.1 (84.0)	0.622 (0.054)
-8.0 to -6.9	68 (23.9)	581.3 (80.9)	0.610 (0.053)	80 (25.3)	591.6 (86.3)	0.617 (0.054)
-6.8 to -5.7	63 (22.1)	590.2 (81.6)	0.608 (0.052)	84 (26.6)	591.6 (78.3)	0.621 (0.052)
≥ -5.6	76 (26.7)	575.7 (72.5)	0.605 (0.053)	74 (23.4)	581.2 (79.2)	0.613 (0.050)
Not measured	857	596.1 (87.0)	0.615 (0.058)	950	604.6 (86.5)	0.625 (0.053)
p (trend)		0.445	0.388		0.256	0.304

Exclusively breastfeeding

duration (months)

Never	36 (3.2)	575.1 (77.0)	0.606 (0.058)	37 (2.9)	622.4 (91.0)	0.633 (0.056)
< 3	400 (35.0)	597.1 (86.4)	0.617 (0.059)	459 (36.3)	602.5 (88.4)	0.625 (0.055)
3 to 5	453 (39.7)	580.2 (80.6)	0.603 (0.053)	478 (37.8)	602.4 (83.9)	0.624 (0.052)
≥ 6	30 (2.6)	604.1 (77.6)	0.617 (0.051)	27 (2.1)	611.1 (86.9)	0.630 (0.055)
Missing	223 (19.5)	606.8 (93.0)	0.622 (0.060)	265 (20.9)	591.6 (82.3)	0.618 (0.051)
p (trend)		0.188	0.045		0.467	0.560

^a Small and large for gestational age were defined as <10th and >90th percentile, respectively, of sex-specific Kramer growth charts [186].

Girls and boys with history of growth problems had decreased bone mineral properties at 7 years of age. In girls, history of asthma was related with increased aBMD but not with BMC. There were no differences in mean BMC and aBMD at age 7 according to history of fracture, and history of gastrointestinal, liver, kidney or musculoskeletal disease. There were also no relevant BMC and aBMD differences between regular use of medication, number of medical or dentist appointments, number of visits to emergency departments or hospitalizations in the previous year, with the exception of a borderline increase in aBMD in girls that had been hospitalized once or more in the previous year, in comparison with those without hospitalizations. Increased mean bone mineral properties were found in children accessing hospital clinics for routine care, in comparison to those followed by a general practitioner from the National Health Service or by a private doctor, but this crude association was observed in boys only (Table 5).

Table 5. Mean (SD) bone mineral content (g) and areal density (g/cm²) at 7 years of age, in girls and boys, according to disease history and health care use

		Girls			Boys		
		n (%)	BMC	aBMD	n (%)	BMC	aBMD
Fracture							
	No	1077 (94.3)	591.9 (85.1)	0.612 (0.057)	1193 (94.2)	600.6 (85.9)	0.612 (0.057)
	Yes	61 (5.3)	591.6 (96.2)	0.612 (0.063)	68 (5.4)	605.2 (80.3)	0.612 (0.063)
	Missing	4 (0.4)	559.4 (69.4)	0.591 (0.039)	5 (0.4)	621.5 (72.6)	0.591 (0.039)
	p (t test)		0.974	0.915		0.667	0.514
Asthma							
	No	1071 (93.8)	590.8 (85.9)	0.611 (0.057)	1177 (93.0)	601.6 (85.4)	0.624 (0.053)
	Yes	62 (5.4)	606.6 (82.6)	0.629 (0.056)	77 (6.1)	597.1 (88.8)	0.624 (0.058)
	Missing	9 (0.8)	609.8 (70.1)	0.624 (0.062)	12 (1.0)	559.5 (81.0)	0.604 (0.058)
	p (t test)		0.157	0.020		0.657	0.960
Growth problems							
	No	1127 (98.7)	592.7 (85.3)	0.613 (0.057)	1251 (98.8)	601.5 (85.4)	0.613 (0.057)
	Yes	10 (0.9)	493.8 (84.3)	0.553 (0.058)	6 (0.5)	503.7 (96.5)	0.553 (0.058)
	Missing	5 (0.4)	576.3 (45.2)	0.605 (0.026)	9 (0.7)	584.5 (66.2)	0.605 (0.026)
	p (t test)		<0.001	<0.001		0.005	0.002

**Gastrointestinal
problems**

No	1074 (94.0)	592.0 (86.1)	0.612 (0.057)	1200 (94.8)	601.8 (85.5)	0.612 (0.057)
Yes	62 (5.4)	586.2 (78.9)	0.611 (0.057)	63 (5.0)	588.0 (85.4)	0.611 (0.057)
Missing	6 (0.5)	621.9 (69.7)	0.642 (0.058)	3 (0.2)	539.4 (82.6)	0.642 (0.058)
p (t test)		0.604	0.868		0.212	0.186

**Kidney or liver
problems**

No	1090 (95.4)	591.9 (85.9)	0.612 (0.057)	1179 (93.1)	600.1 (85.7)	0.623 (0.053)
Yes	41 (3.6)	589.9 (79.0)	0.608 (0.053)	73 (5.6)	617.6 (76.6)	0.632 (0.050)
Missing	11 (1.0)	589.9 (88.7)	0.610 (0.063)	14 (1.1)	584.1 (109.3)	0.606 (0.061)
p (t test)		0.883	0.669		0.088	0.172

**Musculoskeletal
problems**

No	1087 (95.2)	592.3 (86.2)	0.612 (0.057)	1213 (95.8)	601.7 (85.3)	0.612 (0.057)
Yes	42 (3.7)	581.1 (74.2)	0.603 (0.051)	38 (3.0)	580.9 (79.2)	0.603 (0.051)
Missing	13 (1.1)	587.3 (69.8)	0.612 (0.050)	15 (1.2)	591.0 (118.7)	0.612 (0.050)
p (t test)		0.408	0.271		0.138	0.127

**Regular use of any
medication or
supplementation**

No	975 (85.4)	593.4 (85.7)	0.613 (0.057)	1043 (82.4)	602.2 (85.3)	0.613 (0.057)
Yes	150 (13.1)	580.8 (84.0)	0.604 (0.054)	211 (16.7)	596.4 (87.9)	0.604 (0.054)
Missing	17 (1.5)	596.1 (90.3)	0.617 (0.063)	12 (1.0)	572.5 (59.1)	0.617 (0.063)
p (t test)		0.094	0.073		0.375	0.389

**Hospitalizations in the
previous year**

0	1076 (94.2)	590.7 (85.8)	0.611 (0.057)	1169 (92.3)	601.9 (85.7)	0.624 (0.053)
≥1	60 (5.2)	609.2 (82.5)	0.627 (0.055)	93 (7.4)	587.7 (83.3)	0.616 (0.051)
Missing	6 (0.5)	615.7 (65.1)	0.628 (0.050)	4 (0.3)	628.8 (78.8)	0.641 (0.043)
p (trend)		0.104	0.044		0.122	0.161

**Medical appointments
in the previous year**

0	105 (9.2)	589.2 (94.2)	0.610 (0.061)	90 (7.1)	614.4 (78.3)	0.632 (0.048)
1	340 (29.8)	585.8 (80.7)	0.608 (0.056)	352 (27.8)	598.7 (83.6)	0.622 (0.053)
2	245 (21.4)	597.2 (90.2)	0.618 (0.059)	288 (22.8)	603.1 (86.9)	0.623 (0.053)
3	162 (14.2)	589.5 (84.6)	0.609 (0.056)	189 (14.9)	595.7 (87.8)	0.622 (0.055)
≥4	286 (25.0)	596.0 (84.7)	0.615 (0.056)	343 (27.1)	601.3 (87.1)	0.625 (0.054)
Missing	4 (0.4)	631.0 (69.9)	0.626 (0.044)	4 (0.3)	553.1 (59.2)	0.605 (0.042)
p (trend)		0.184	0.203		0.528	0.965

**Dentist appointments in
the previous year**

0	341 (29.9)	598.1 (94.6)	0.616 (0.061)	440 (34.8)	595.4 (83.5)	0.622 (0.053)
1	363 (31.8)	588.3 (76.7)	0.611 (0.053)	388 (30.6)	603.7 (82.0)	0.625 (0.051)
2	217 (19.0)	592.2 (87.4)	0.612 (0.059)	200 (15.8)	601.3 (89.5)	0.623 (0.054)
≥3	212 (18.6)	586.3 (83.5)	0.607 (0.054)	227 (17.9)	606.0 (91.8)	0.624 (0.056)
Missing	9 (0.8)	614.3 (67.7)	0.624 (0.050)	11 (0.9)	615.8 (87.6)	0.640 (0.052)
p (trend)		0.228	0.091		0.255	0.924

**Visits to emergency
department in the
previous year**

0	509 (44.6)	590.9 (85.0)	0.611 (0.057)	499 (39.4)	603.6 (87.3)	0.625 (0.055)
1	294 (25.7)	596.1 (87.5)	0.614 (0.059)	361 (28.5)	602.4 (81.5)	0.624 (0.050)
2	164 (14.4)	585.2 (82.4)	0.608 (0.054)	198 (15.6)	595.6 (88.7)	0.621 (0.054)
≥3	156 (13.7)	591.2 (88.5)	0.613 (0.058)	187 (14.8)	597.2 (84.1)	0.621 (0.052)
Missing	19 (1.7)	611.2 (77.4)	0.637 (0.052)	21 (1.7)	597.3 (99.4)	0.625 (0.057)
p (trend)		0.818	0.999		0.363	0.514

Healthcare provision

NHS GP ^a	767 (67.2)	591.7 (86.0)	0.613 (0.058)	787 (62.2)	603.0 (82.2)	0.625 (0.051)
Private doctor	246 (21.5)	592.1 (82.4)	0.611 (0.055)	345 (27.2)	590.1 (86.9)	0.616 (0.052)
Hospital clinic	66 (5.8)	587.6 (92.7)	0.608 (0.058)	69 (5.4)	617.6 (104.0)	0.636 (0.066)
Other	3 (0.3)	546.5 (23.1)	0.581 (0.011)	5 (0.4)	571.6 (54.8)	0.604 (0.041)
Missing	60 (5.3)	598.3 (88.8)	0.614 (0.061)	60 (4.7)	619.9 (92.8)	0.641 (0.061)
p (ANOVA)		0.805	0.696		0.028	0.008

^a National Health Service General practitioners

In girls, there were no clear crude associations between bone mineral properties and physical activity. In boys, mean bone mineral properties increased with increasing time spent in sports practice and with increasing time spent watching TV on weekend days, although the last relationship was not consistent for the two measures of bone mineralization (Table 6).

Table 6. Mean (SD) bone mineral content (g) and areal density (g/cm²) at 7 years of age, in girls and boys, according to physical activity

		Girls			Boys		
	n (%)	BMC	aBMD		n (%)	BMC	aBMD
Time spent watching TV per weekday (minutes)							
< 30	123 (10.8)	610.0 (91.5)	0.625 (0.059)		110 (8.7)	589.4 (100.9)	0.618 (0.064)
30 to 59	346 (30.3)	584.8 (79.2)	0.608 (0.054)		341 (26.9)	600.9 (83.6)	0.623 (0.050)
60 to 119	509 (44.6)	592.0 (87.8)	0.611 (0.058)		582 (46.0)	601.2 (84.2)	0.624 (0.052)
≥ 120	157 (13.8)	591.3 (85.9)	0.614 (0.058)		229 (18.1)	606.1 (84.0)	0.627 (0.054)
Missing	7 (0.6)	609.6 (92.2)	0.629 (0.059)		4 (0.3)	597.2 (93.2)	0.633 (0.056)
p (trend)		0.346	0.628			0.174	0.218
Time spent watching TV per weekend day (minutes)							
< 60	78 (6.8)	594.6 (92.6)	0.614 (0.060)		64 (5.1)	572.5 (89.6)	0.605 (0.058)
60 to 119	294 (25.7)	590.4 (83.8)	0.612 (0.058)		255 (20.1)	603.5 (86.2)	0.625 (0.052)
120 to 179	369 (32.3)	586.8 (84.4)	0.608 (0.057)		380 (30.0)	602.7 (85.3)	0.624 (0.052)
≥ 180	388 (34.0)	597.1 (87.0)	0.616 (0.057)		557 (44.0)	602.0 (84.5)	0.625 (0.053)
Missing	13 (1.1)	593.3 (76.0)	0.610 (0.045)		10 (0.8)	591.6 (94.8)	0.624 (0.071)
p (trend)		0.567	0.366			0.056	0.036
Time spent in active play per weekday (minutes)							
< 30	414 (36.2)	596. 6 (85.0)	0.615 (0.056)		415 (32.8)	606.0 (88.4)	0.626 (0.056)
30 to 59	251 (22.0)	588.9 (76.7)	0.611 (0.055)		257 (20.3)	600.9 (86.1)	0.624 (0.052)
60 to 119	346 (30.3)	586.7 (90.4)	0.609 (0.060)		409 (32.3)	594.0 (82.8)	0.619 (0.052)
≥ 120	123 (10.8)	596.8 (91.6)	0.615 (0.055)		175 (13.8)	605.1 (84.0)	0.626 (0.049)
Missing	8 (0.7)	581.4 (74.3)	0.608 (0.057)		10 (0.8)	601.3 (89.6)	0.627 (0.055)
p (trend)		0.210	0.327			0.194	0.249

**Time spent in active play
per weekend day (minutes)**

< 60	106 (9.3)	592.6 (81.1)	0.612 (0.055)	92 (7.3)	610.6 (98.1)	0.630 (0.064)
60 to 119	254 (22.2)	587.8 (81.4)	0.609 (0.055)	263 (20.8)	601.9 (85.2)	0.624 (0.054)
120 to 179	316 (27.7)	591.0 (92.4)	0.611 (0.061)	374 (29.5)	603.5 (88.6)	0.626 (0.055)
≥ 180	452 (39.6)	594.5 (84.7)	0.614 (0.056)	527 (41.6)	597.3 (81.2)	0.621 (0.049)
Missing	14 (1.2)	591.7 (70.7)	0.611 (0.042)	10 (0.8)	581.7 (82.0)	0.614 (0.055)
p (trend)		0.587	0.432		0.159	0.190

**Time spent in sports
practice per week (minutes)**

< 60	101 (8.8)	581.5 (93.5)	0.603 (0.060)	140 (11.1)	599.9 (92.9)	0.624 (0.060)
60 to 179	530 (46.4)	592.3 (87.3)	0.612 (0.057)	508 (40.1)	596.3 (79.6)	0.621 (0.049)
180 to 239	230 (20.1)	594.0 (78.4)	0.614 (0.054)	233 (18.4)	596.3 (87.8)	0.620 (0.054)
≥ 240	256 (22.4)	593.6 (85.5)	0.615 (0.058)	357 (28.2)	612.8 (89.5)	0.631 (0.055)
Missing	25 (2.2)	585.0 (84.9)	0.615 (0.055)	28 (2.2)	577.8 (67.7)	0.606 (0.043)
p (trend)		0.267	0.106		0.032	0.022

No clear dose-response relations between daily calcium, vitamin D or energy intakes and BMC or aBMD in Generation XXI children were observed (Table 7).

Table 7. Mean (SD) bone mineral content (g) and areal density (g/cm²) at 7 years of age, in girls and boys, according to calcium, vitamin D and total energy intakes

		Girls			Boys		
		n (%)	BMC	aBMD	n (%)	BMC	aBMD
Calcium intake (mg/day)							
< 800	348 (30.5)	585.9 (83.7)	0.608 (0.056)	328 (25.9)	600.9 (84.6)	0.622 (0.053)	
800 to 999	311 (27.2)	593.9 (89.7)	0.613 (0.060)	297 (23.5)	597.0 (85.2)	0.623 (0.052)	
1000 to 1199	211 (18.5)	596.4 (83.2)	0.616 (0.056)	293 (23.1)	596.5 (85.4)	0.620 (0.054)	
≥ 1200	240 (21.0)	591.7 (83.7)	0.612 (0.056)	322 (25.4)	608.8 (87.3)	0.629 (0.054)	
Missing	32 (2.8)	605.6 (95.2)	0.620 (0.060)	26 (2.0)	599.0 (80.4)	0.623 (0.042)	
p (trend)		0.193	0.156		0.342	0.210	
Vitamin D intake (µg/day)							
< 2.0	402 (35.2)	594.7 (87.7)	0.614 (0.060)	391 (30.9)	598.3 (87.2)	0.623 (0.054)	
2.0 to 2.4	360 (31.5)	586.7 (81.3)	0.610 (0.055)	412 (32.5)	607.7 (85.7)	0.628 (0.052)	
≥ 2.5	348 (30.5)	592.5 (84.3)	0.612 (0.056)	437 (34.5)	597.1 (84.2)	0.620 (0.053)	
Missing	32 (2.8)	605.6 (95.2)	0.620 (0.059)	26 (2.0)	599.0 (80.4)	0.623 (0.043)	
p (trend)		0.976	0.812		0.978	0.812	
Total energy intake (kcal/day) ^a							
< 1400	214 (18.7)	591.8 (89.3)	0.612 (0.060)	195 (15.4)	602.6 (96.8)	0.626 (0.060)	
1400 to 1599	263 (23.0)	596.7 (90.3)	0.616 (0.060)	238 (18.8)	595.8 (80.8)	0.620 (0.050)	
1600 to 1999	466 (40.8)	590.2 (80.7)	0.612 (0.054)	560 (44.2)	601.4 (82.9)	0.624 (0.051)	
≥ 2000	167 (14.6)	585.9 (84.9)	0.606 (0.055)	247 (19.5)	603.9 (87.5)	0.626 (0.056)	
Missing	32 (2.8)	605.6 (95.2)	0.620 (0.060)	26 (2.0)	599.0 (80.4)	0.623 (0.043)	
p (trend)		0.576	0.361		0.590	0.693	

^a Using the USDA's Guidelines (U.S. Department of Agriculture & U.S. Department of Health and Human Services, 2010) estimated energy requirements for moderately active children aged 4 to 6 years (1400–1600 kcal).

4.2. Gestational Weight Gain and Offspring Bone Mass: Different Associations in Healthy Weight versus Overweight Women

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Gestational Weight Gain and Offspring Bone Mass: Different Associations in Healthy Weight Versus Overweight Women

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ABSTRACT

Weight management strategies during pregnancy reduce child cardiometabolic risk. However, because maternal weight has an overall positive correlation with offspring bone mass, pregnancy weight management could adversely affect child bone health. We aimed to estimate associations between gestational weight gain (GWG) and bone mineralization in the offspring at 7 years of age, and test early pregnancy body mass index (BMI) as an effect modifier. We analyzed prospective data from 2167 mother-child pairs from the Generation XXI birth cohort who underwent whole-body dual-energy X-ray absorptiometry at 7 years of age. GWG was analyzed as a continuous measure and using the Institute of Medicine categories. In the whole sample and for each early pregnancy BMI category (under/normal weight and overweight/obese), relationships between GWG and offspring bone measures (bone mineral content [BMC], bone areal density [aBMD], size-corrected BMC [scBMC], and height) at 7 years were fitted through local polynomial regression and smoothing splines. The magnitude of associations was estimated through linear regression coefficients (95% CIs), crude and adjusted for maternal age, height, educational level, and child gestational age. In under/normal weight mothers, GWG was associated with slightly increased bone measures at 7 years (per 5 kg of GWG, BMC: 0.07 SD [95% CI, 0.01 to 0.12]; aBMD: 0.10 SD [95% CI, 0.05 to 0.15], scBMC: 0.11 SD [95% CI, 0.06 to 0.16], and height: 0.05 SD [95% CI, 0.00 to 0.10]), while in overweight/obese mothers no effect of GWG on bone was observed (BMC: 0.02 SD [95% CI, -0.04 to 0.09]; aBMD: 0.02 SD [95% CI, -0.04 to 0.08], scBMC: 0.01 SD [95% CI, -0.06 to 0.08], and height: 0.02 SD [95% CI, -0.04 to 0.08]). Also, no advantageous effect of gaining weight above the Institute of Medicine recommendations was observed in either early pregnancy BMI group. Our results suggest that adherence to Institute of Medicine recommendations for pregnancy weight gain is unlikely to have a negative repercussion on offspring bone health, particularly in women with excess weight in early pregnancy. © 2018 American Society for Bone and Mineral Research.

KEY WORDS: GENERATION XXI; COHORT STUDY; BONE DENSITY; GESTATIONAL WEIGHT GAIN; BODY MASS INDEX

Introduction

The weight gained by women during pregnancy is an important and potentially modifiable determinant of short-term and long-term health outcomes for both the mother and the child.⁽¹⁾ Because its management is a key component of prenatal care, in 2009, the US Institute of Medicine (IOM) published revised guidelines for maternal gestational weight gain (GWG) according to early pregnancy body mass index (BMI) categories, where an overall inverse relation between baseline BMI and weight gain is recommended.^(1–3) Those guidelines are now used as standard recommendations in the majority of

high-income countries. Due to their policy nature, IOM guidelines overlook the heterogeneity of the associations between GWG and distinct health outcomes in terms of strength and direction.⁽⁴⁾ For instance, although an inverted U-shaped association has been suggested for the effect of GWG on preterm birth, a monotonic dose-response association was described for GWG and birth weight, after adjustment for gestational age.⁽¹⁾ Heterogeneous associations have also been found for long-term child health outcomes, namely adiposity, cardiometabolic risk, and asthma.⁽⁵⁾

With regard to bone mass, there is evidence of positive effects of GWG on offspring bone mineral content (BMC) and areal

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density (aBMD).^(6,7) By altering the intrauterine environment, including nutrient availability or endocrine factors such as leptin and estrogen, GWG may influence not only fetal bone formation, but also the programming of bone strength during childhood and even later in life.^(8,9) However, it seems plausible that the effect of GWG on offspring bone is qualitatively or quantitatively modified by prepregnancy maternal weight status. Particularly in view of the IOM guidelines for GWG—that recommend lower and narrower ranges of weight gain for overweight/obese women—possible implications of following those recommendations for the offspring's bone mineralization should be assessed and compared by prepregnancy BMI groups.

By means of a longitudinal study of children and their mothers within the Generation XXI birth cohort, our objective was to quantify the associations between GWG, continuously and using IOM recommended categories, and dual-energy X-ray absorptiometry (DXA)-derived bone measures and height of the offspring at 7 years of age. Specifically, we aimed to explore a possible modification of the effect of GWG on child bone mineralization by maternal early pregnancy BMI.

Materials and Methods

Generation XXI cohort assembly and follow-up

This study was based on the Generation XXI birth cohort whose full details have been published.^(10,11) In 2005 to 2006, all women hospitalized for childbirth in one of the five public maternity units of Porto, Portugal, whose obstetric outcome was a live birth with at least 24 weeks of gestation, were eligible to participate. Of the invited mothers, 91.4% ($n = 8495$ women) accepted to participate and their 8647 infants were enrolled in the cohort study. In the 7-year-old follow-up wave (2012/2014), 67.6% of the cohort (5849 children) was reevaluated by face to face interview. This age represents as a biological milestone before the onset of puberty, when sexual development becomes a major driving force for growth and development, particularly regarding microarchitectural and macroarchitectural changes to the skeleton. Details of the cohort design and description of the cohort's baseline characteristics were provided as Supporting Data and Supporting Table 1.

The study protocol was approved by the Ethics Committee of Hospital de São João and registered with the Portuguese Authority of Data Protection and was carried out in accordance with the principles of the Declaration of Helsinki. In all evaluations written informed consent was obtained from parents or legal guardians and oral assent from children.

Measures of early pregnancy BMI and GWG

At baseline, within 72 hours after delivery, trained interviewers applied face-to-face structured questionnaires including data on demographic and socioeconomic characteristics, lifestyles, medical history, and anthropometrics. In the context of this large cohort comprising women recruited after delivery, the preferred method to collect weight throughout pregnancy was self-report by women. Besides feasibility, this option is supported by a previous systematic review showing that weight misreporting does not largely bias associations between pregnancy-related weight and birth outcomes.⁽¹²⁾ To calculate early pregnancy BMI and GWG we used weight in early pregnancy as the baseline measure, defined as the self-reported weight in kilograms at the beginning of pregnancy

("What was your weight at the beginning of pregnancy?") or on the first prenatal medical visit ("What was your weight on the first pregnancy appointment?") if the latter occurred before the 13th gestational week. For women with missing information on this variable (6.0%), early pregnancy weight was recovered using either obstetric clinical records (0.6%) or the proxy question "What was your weight in the 2 years preceding pregnancy?" (5.4%). Clinical record review was planned with the purpose of recovering only data that were missing from the baseline questionnaire, which is why anthropometrics from this source is available only for a small number of participants.⁽¹³⁾ Sensitivity analysis to assess the impact of using these different sources of information is described below. Predelivery weight was also obtained from the questionnaire ("What was your weight at the end of pregnancy?") or recovered from clinical records in the 3.1% of mothers with missing questionnaire information. Height was measured by the interviewer to the nearest 0.1 cm (55.4% of the mothers), abstracted from the national ID card when measurement was not possible (38.7% of the mothers) or recovered from clinical records (6.0% of the mothers). Early pregnancy BMI was calculated (weight/height [kg/m²]) and categorized according to the standard World Health Organization (WHO) definition: underweight (<18.5 kg/m²), normal (18.5 to 24.9 kg/m²), overweight (25.0 to 29.9 kg/m²), or obese (≥ 30.0 kg/m²).⁽¹⁴⁾

GWG was computed as the difference between the mother's self-reported predelivery weight and her early pregnancy weight. For 0.6% of the participants, GWG was recovered from the clinical record review. Women were categorized as gaining below (insufficient GWG), as recommended (adequate GWG), or above (excessive GWG) the IOM recommendations, as detailed in Table 1 footnote c.⁽¹⁾

Child bone densitometry and anthropometric data

In the follow-up evaluation at the age of 7 years, all children assessed between December 1, 2012, and August 31, 2013, were consecutively invited to undergo a whole-body DXA scan (3015 children, 43.8% of the participants). Scans were performed using a Hologic Discovery QDR[®] 4500W device (software version 13.3.0.1; Hologic Inc., Bedford, MA, USA) according to standard manufacturer's protocol. Standard quality assurance tests were performed daily using the spine phantom according to manufacturer's instructions. The coefficient of variation obtained from repeated phantom measurements was below 1%. Scans were evaluated immediately after acquisition and later validated by a second technician with at least 5 years of experience. Total body less head BMC (g) and aBMD (g/cm²) were obtained.⁽¹⁵⁾ Size-corrected BMC (scBMC) was derived separately for girls and boys by linear regression of BMC on bone area and addition of the residuals of the regression to the mean sample BMC.⁽¹⁶⁾ Weight and height were measured according to standard procedures and BMI was calculated. Height was also used in the present study as an outcome measure, as a proxy of linear growth of long bones.

Maternal and offspring neonatal data

Maternal age at delivery was recorded. Maternal educational level at the baseline evaluation was recorded as the number of completed years of education. Clinical records were reviewed at birth to retrieve data on birth weight and gestational age of the offspring.

Table 1. Maternal and Child Characteristics According to Groups of Early Pregnancy BMI

Characteristic	Whole sample (n = 2167)	Early pregnancy BMI groups ^a		p ^b
		Under/normal weight (n = 1392)	Overweight/obese (n = 775)	
Maternal characteristics				
Early pregnancy BMI (kg/m ²), mean ± SD	24.4 ± 4.4	21.8 ± 1.9	28.9 ± 3.8	<0.001
Age at delivery (years), mean ± SD	29.9 ± 5.1	29.5 ± 5.0	30.7 ± 5.1	<0.001
Women by educational level, n (%)				<0.001
≤9 years	859 (39.7)	491 (35.3)	368 (47.5)	
10–12 years	624 (28.8)	385 (27.7)	239 (30.8)	
>12 years	682 (31.5)	514 (37.0)	168 (21.7)	
Missing	2	2	0	
Weight gain during pregnancy (kg), mean ± SD	13.2 ± 5.3	14.1 ± 4.9	11.7 ± 5.6	<0.001
Women by categories of gestational weight gain, n (%) ^c				<0.001
Insufficient	513 (23.7)	406 (29.2)	107 (13.8)	
Adequate	861 (39.7)	614 (44.1)	247 (31.9)	
Excessive	793 (36.6)	372 (26.7)	421 (54.3)	
Women with gestational diabetes, n (%)	126 (5.8)	58 (4.2)	68 (8.8)	<0.001
Missing	6	4	2	
Women who ever smoked during pregnancy, n (%)	450 (20.9)	313 (22.6)	137 (17.9)	0.010
Missing	15	7	8	
Child characteristics				
Gestational age at birth (weeks), mean ± SD	38.7 ± 1.6	38.7 ± 1.6	38.8 ± 1.6	0.184
Birth weight (g), mean ± SD	3197.6 ± 475.8	3167.0 ± 473.4	3252.5 ± 475.3	<0.001
Weight at 7 years, mean ± SD				
Crude (kg)	27.3 ± 5.5	26.4 ± 4.8	29.0 ± 6.2	<0.001
Z-score, WHO reference ^d	0.67 ± 1.14	0.48 ± 1.09	1.01 ± 1.17	<0.001
Z-score, Generation XXI reference ^e	0.02 ± 0.99	−0.15 ± 0.87	0.32 ± 1.10	<0.001
Height at 7 years, mean ± SD				
Crude (cm)	124.7 ± 5.6	124.4 ± 5.6	125.2 ± 5.6	0.002
Z-score, WHO reference ^d	0.30 ± 0.94	0.24 ± 0.94	0.40 ± 0.93	0.002
Z-score, Generation XXI reference ^e	−0.03 ± 0.99	−0.10 ± 0.99	0.08 ± 0.98	<0.001
BMI at 7 years, mean ± SD				
Crude (kg/m ²)	17.4 ± 2.6	17.0 ± 2.2	18.3 ± 3.0	<0.001
Z-score, WHO reference ^d	0.68 ± 1.16	0.47 ± 1.08	1.06 ± 1.21	<0.001
Z-score, Generation XXI reference ^e	0.05 ± 0.99	−0.14 ± 0.84	0.38 ± 1.13	<0.001
Child DXA-derived bone measures at 7 years				
BMC, mean ± SD				
Crude (g)	597.4 ± 86.3	588.8 ± 84.2	612.7 ± 87.8	<0.001
Z-score, Zemel and colleagues (2011) ⁽¹⁷⁾ reference ^f	−0.04 ± 0.89	−0.14 ± 0.89	0.14 ± 0.86	<0.001
Z-score, Generation XXI reference ^g	0.00 ± 1.00	−0.10 ± 0.98	0.18 ± 1.02	<0.001
aBMD, mean ± SD				
Crude (g/cm ²)	0.619 ± 0.056	0.612 ± 0.054	0.631 ± 0.056	<0.001
Z-score, Zemel and colleagues (2011) ⁽¹⁷⁾ reference ^f	0.40 ± 1.09	0.26 ± 1.07	0.66 ± 1.08	<0.001

continued

continued

Table 1. (Continued)

Characteristic	Whole sample (n = 2167)	Early pregnancy BMI groups ^a	
		Under/normal weight (n = 1392)	Overweight/obese (n = 775)
Z-score, Generation XXI reference ^a	0.00 ± 1.00	-0.12 ± 0.97	0.22 ± 1.02
scBMC, mean ± SD	597.4 ± 39.6	592.9 ± 36.8	605.4 ± 43.1
Z-score, Generation XXI reference ^a	0.00 ± 1.00	-0.12 ± 0.93	0.21 ± 1.09

BMI = body mass index; BMC = bone mineral content; aBMD = areal bone mineral density; scBMC = size-corrected bone mineral content; GWG = gestational weight gain

^aAccording to standard WHO definition.

^bValues of *p* obtained for comparison between early pregnancy BMI groups (Wilcoxon rank or chi-square tests).

^cAccording to the Institute of Medicine classification (2009): Insufficient: GWG <12.5 kg for underweight women, <11.5 kg for normal-weight women, <7 kg for overweight women, and <5 kg for obese women.

Adequate: GWG of 12.5–18 kg for underweight women, 11.5–16 kg for normal-weight women, 7–11.5 kg for overweight women, and 5–9 kg for obese women. Excessive: GWG >18 kg for underweight women, >16 kg for normal-weight women, >11.5 kg for overweight women, and >9 kg for obese women.

^dAge and sex-specific Z-scores computed using WHO reference data.⁽¹⁹⁾

^eAge and sex-specific Z-scores computed based on the means and SDs derived from all Generation XXI participants in the 7-year-old follow-up wave with anthropometric data (n = 5838).

^fAge and sex-specific Z-scores computed using the Bone Mineral Density in Childhood Study reference data.⁽¹⁷⁾

^gAge and sex-specific Z-scores computed based on the means and SDs derived from the study sample (n = 2167).

Study sample

Of the 3015 children eligible for bone densitometry, 2408 had a valid DXA scan (79.9% of the eligible subsample) after excluding participants who refused to perform the scan and those whose images had unacceptable technical quality. For the current analysis, we additionally excluded children from multiple pregnancies (n = 111) and participants with no information on maternal GWG (n = 115) or without information to compute maternal early pregnancy BMI (n = 15) (Supporting Fig. 1). Additionally, we analyzed implausible values of GWG (less than 0 kg and more than 30 kg) and for six participants we recovered weights from an alternative source of information, among those previously described. The final sample comprised 2167 mother-child pairs (46.8% girls) whose comparison with the remainder of the cohort is presented in Supporting Table 2, which shows that study participants were more likely to present higher socioeconomic position than nonparticipants.

Data analysis

Given the frequency of underweight (3.1%) and obese women (10.4%), we combined the four early pregnancy BMI categories into two groups: (i) under/normal weight, and (ii) overweight/obese. Sensitivity analyses were conducted to test the robustness of that option. Maternal and child characteristics were compared between early pregnancy BMI groups with Wilcoxon rank-sum or chi-square tests. Age- and sex-specific Z-scores were computed for BMC and aBMD based on the method and reference values published by Zemel and colleagues.⁽¹⁷⁾ We also computed internal Z-scores based on the means and SDs derived from the study sample (n = 2167), which was essential to obtain scBMC Z-scores. Age- and sex-specific weight, height, and BMI Z-scores were obtained according to WHO growth charts⁽¹⁸⁾ and also using as reference all Generation XXI participants with anthropometric data at 7 years of age (n = 5838).

The shapes of the associations between GWG and bone parameters were assessed using local polynomial regression and smoothing splines (R packages *fANCOVA* and *splines*; R Foundation for Statistical Computing, Vienna, Austria; <https://www.r-project.org/>). Because the visual inspection of plots was consistent with linear relations, estimates of the magnitude of the associations between GWG and DXA-derived bone measures and height were obtained by using multiple linear regression coefficients and respective 95% confidence intervals (95% CIs). Results are presented per 5 kg increase in GWG to improve readability.

To assess whether the relationship between GWG and offspring bone mineralization was modified by maternal weight status, we stratified regression analyses by early pregnancy BMI groups. Analyses were conducted crude and adjusted for potential confounding factors, defined as documented causes of both GWG and child's bone mineralization and that are not likely to mediate their relationship: maternal age, height and educational level, and offspring gestational age.

Additional analyses with GWG defined as a categorical variable according to IOM categories were conducted by computing adjusted means of the offspring's BMC, aBMD, scBMC, and height using analysis of covariance (ANCOVA), adjusted for the previously defined potential confounders. The differences (95% CI) between those means in adequate versus insufficient and excessive versus adequate weight gain groups were estimated using Stata's postestimation command *lincom* (Stata Corporation, Inc., College Station, TX, USA).

Sensitivity analyses

To assess the effect of including the 6.0% of women whose early pregnancy weight was recovered from the alternative sources described (ie, clinical records or an alternative item on the questionnaire), we carried out a sensitivity analysis by recomputing estimates after excluding this group, and comparing these estimates with those obtained for the whole sample. Additionally, to assess the impact of misclassification due to self-reporting of weight, we recalculated estimates after excluding women classified as having higher susceptibility to weight underreporting according to published literature⁽¹⁹⁻²³⁾, ie, participants with at least one of the following characteristics ($n = 641$ mothers): young maternal age (<25 years old), low educational level (<6 years), unmarried, late prenatal care (after the 12th gestational week), and higher parity (two or more previous pregnancies).

Statistical analysis and graphics were performed using Stata version 11.2 for Windows (Stata Corp) and R version 3.5.1 (The R Foundation for Statistical Computing).

Results

Maternal and child characteristics are presented in Table 1. At the beginning of pregnancy, 35.8% of the mothers were overweight or obese. In the whole sample, the mean $\pm$ SD maternal GWG was 13.2 ± 5.3 kg, greater in under/normal weight women than in women with excess weight (14.1 versus 11.7 kg, $p < 0.001$). More than one-third (36.6%) of children were born to women who gained excessive weight during pregnancy, according to the IOM recommendations, whereas 23.7% to women who had insufficient GWG. Women with higher BMI in early pregnancy had children with increased BMC, aBMD and scBMC at 7 years of age.

Fig. 1A–D presents the relationship between GWG and DXA-derived bone measures and height in the whole sample, including the plots of local polynomial regressions and smoothing splines, whereas Fig. 2A–D shows those relationships after stratification by early pregnancy BMI. When compared to smoothing splines, local polynomial regression showed higher variance in the ends of the GWG spectrum, as expected due to data sparsity. In under/normal weight mothers, visual inspection of the association between GWG and offspring bone suggests linear relations for all outcomes. After fitting linear regression models we estimated a modest positive slope, which persisted after adjustment for maternal age, height, educational level, and gestational age (β [95% CI]: BMC Z-score = 0.07 [95% CI, 0.01 to 0.12] per 5-kg weight gain; aBMD Z-score = 0.10 [95% CI, 0.05 to 0.15] per 5 kg; scBMC Z-score = 0.11 [95% CI, 0.06 to 0.16] per 5 kg; height Z-score = 0.05 [95% CI, 0.00 to 0.10] per 5 kg) (Fig. 3A–D). Nevertheless, the predictive performance of the model was low, with r^2 estimates between 1% and 2% for all bone measures. More important, however, among women with excess weight in early pregnancy, there were no linear associations between GWG and offspring DXA-derived bone measures or height (Fig. 2A–D), before or after adjustment for confounders (adjusted β [95% CI]: BMC Z-score = 0.02 [95% CI, -0.04 to 0.09] per 5-kg weight gain; aBMD Z-score = 0.02 [95% CI, -0.04 to 0.08] per 5 kg; and scBMC Z-score = 0.01 [95% CI, -0.06 to 0.08] per 5 kg; height Z-score = 0.02 [95% CI, -0.04 to 0.08]) (Fig. 3A–D). As expected, the fit of the model was very low, with r^2 estimates below 1%.

Within the under/normal weight group, and taking women with adequate GWG as the reference category, women with insufficient GWG had children with aBMD on average 0.14 SD (95% CI, 0.02 to 0.26) lower and scBMC 0.19 SD (95% CI, 0.07 to 0.30) lower at 7 years of age, after adjustment for confounders presented in Table 2. In contrast, in overweight women, no such relation was observed. Interestingly, for both early pregnancy BMI groups, there were no differences in mean bone measures between children born to women with excessive when compared to adequate GWG.

Sensitivity analysis

After restricting the analysis to participants whose information for weight in early pregnancy was collected from the primary source; ie, self-reported weight at the beginning of pregnancy or at the first medical appointment in the first trimester ($n = 2108$ [97.3%] of all mothers; $n = 1357$ [97.6%] of under/normal weight mothers; and $n = 751$ [96.9%] of overweight/obese mothers), the associations between GWG and offspring bone properties remained similar to those obtained including participants with alternative sources of information (Fig. 3A–D). Associations between GWG and offspring bone properties also remained essentially unchanged after restricting the analysis to mothers who were classified as having lower susceptibility to weight misreporting ($n = 1001$ [72.0%] of under/normal weight mothers and $n = 523$ [67.5%] of overweight/obese mothers) (Fig. 3A–D).

Discussion

In the present study, we found a modest direct linear association between maternal GWG and bone mineralization in the offspring of women who were under/normal weight in early pregnancy, possibly reflecting a weak biological effect. However, among overweight/obese women, increased GWG had no apparent relation with offspring bone mineralization. The corresponding causal interpretation would be that there is little or no apparent advantage of gaining excess weight during pregnancy for offspring bone health, particularly in women with excessive weight in early pregnancy. Our results were robust to different bone mineralization measures—DXA-derived or height—and different exposure definitions—GWG as a continuous variable or as IOM categories. Results remained practically unchanged after adjustment for maternal age, height and educational level, and gestational age of the offspring. Likewise, no relevant changes were found after excluding women whose weight was recovered from alternative sources or those more susceptible to weight misreporting.

Previous studies have looked at maternal body size during pregnancy and offspring bone.⁽⁶⁻⁹⁾ Maternal fat stores during pregnancy were positively associated with offspring bone mineral and geometry at birth in the Southampton Women's Survey, particularly in late pregnancy.^(8,9) With respect to weight changes, GWG was an independent predictor of BMD in 6-month-old infants from Generation R and in Chinese children aged 0 to 3 years.^(6,7) In our study, we found evidence of a weak relationship between maternal GWG and childhood bone measures only among under/normal weight women. However, when stratified by IOM categories, there were no differences between mean bone properties between women gaining excessive and adequate weight, in contrast to the comparison between women gaining adequate and insufficient weight. This

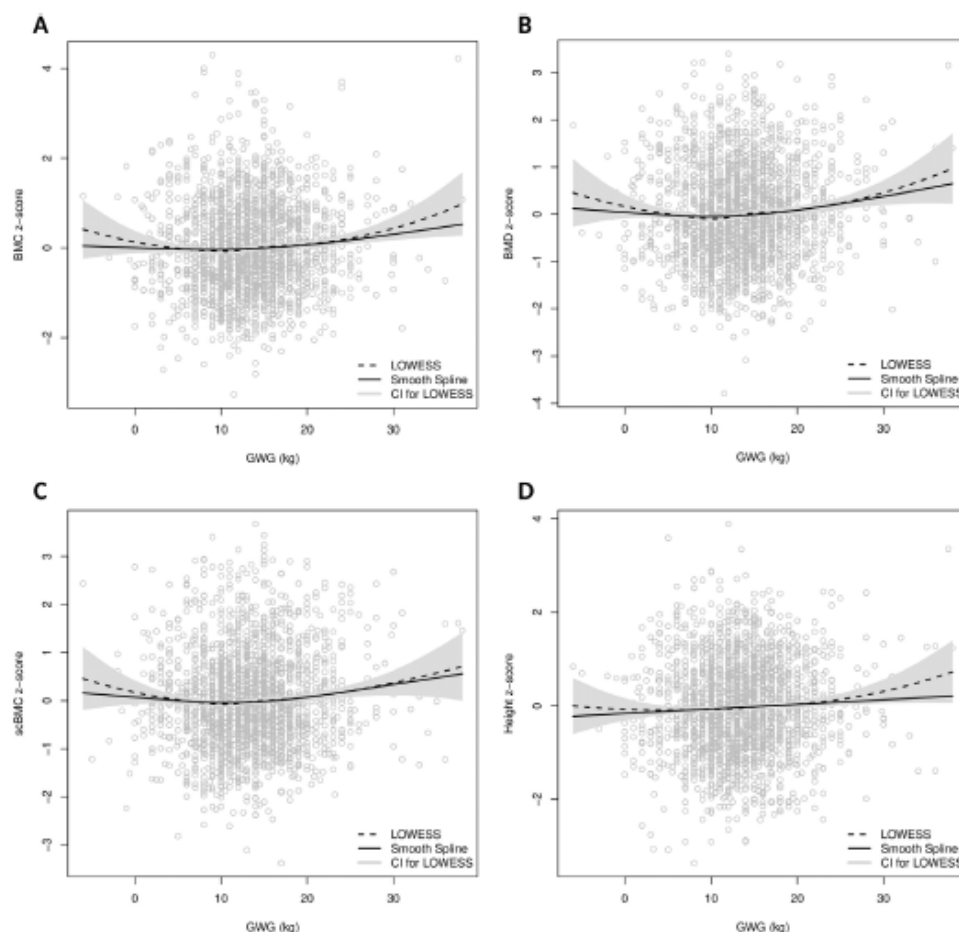


Fig. 1. Local polynomial regression and smoothing splines for gestational weight gain and offspring bone measures at 7 years. BMC (A), aBMD (B), scBMC (C), height (D). Dashed line, local polynomial regression; solid line, smoothing spline. aBMD = areal bone mineral density; BMC = bone mineral content; GWG = gestational weight gain; scBMC = size-corrected bone mineral content.

may be explained by either a relative lack of precision on the higher end of the GWG spectrum or a true leveling off of the biological effect with increasing GWG.

To the best of our knowledge, no studies have looked at the potential effect modification by maternal early pregnancy BMI on GWG-bone associations, even though GWG also showed greater impact on childhood overweight when mothers had normal versus excess weight before pregnancy.⁽²⁴⁻²⁷⁾ Possible intrauterine mechanisms underlying a differential response of pediatric bone to GWG in low/normal BMI versus higher BMI in early pregnancy are several. First, differences in glucose metabolism profiles may contribute to a differential effect. When compared to overweight/obese women, under/normal weight women are less likely to have insulin resistance and/or impaired glucose tolerance during pregnancy,⁽²⁸⁾ and may be more sensitive to weight changes during gestation, inducing a more effective decrease in insulin sensitivity, followed by an augmented supply of nutrients to the fetus and enhanced bone

accretion.⁽²⁹⁾ Another possible mechanism may be related to chronic low-grade systemic inflammation associated with adiposity.^(30,31) The placenta of obese women has increased number of macrophages and enhanced expression of proinflammatory cytokines interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α), and IL-6 in comparison with that of normal weight women⁽³⁰⁾ and low-grade inflammation in the higher BMI group may have restricted bone mineral accretion associated to GWG. Also, obese women are more likely to have low serum levels of vitamin D,⁽³²⁾ whose insufficiency has been associated with reduced bone mineral accrual.⁽³³⁾ Additionally, differences in weight or body composition trajectories according to early pregnancy BMI may also contribute to the discrepancy: in contrast to women with excessive weight at time of conception, higher weight gains during the second and third trimesters are more frequent in normal-weight women.^(1,34) Because 80% of fetal bone mineral accumulation occurs during the last trimester of pregnancy,⁽³⁵⁾ greater weight gains later in pregnancy may

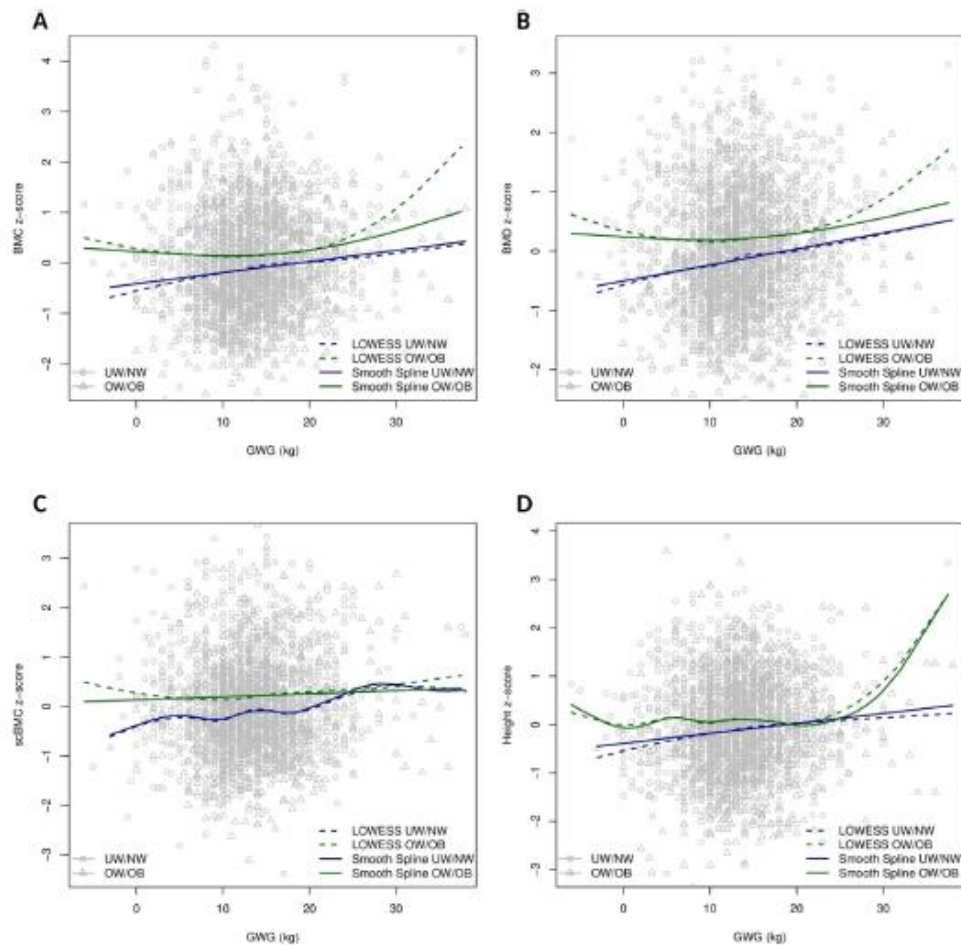


Fig. 2. Local polynomial regression and smoothing splines for gestational weight gain and offspring bone measures at 7 years, in under/normal weight and overweight/obese women. BMC (A), aBMD (B), scBMC (C), height (D). Dashed blue line, local polynomial regression in under/normal weight women; solid blue line, smoothing spline in under/normal weight women; dashed green line, local polynomial regression in overweight/obese women; solid green line, smoothing spline in overweight/obese women. aBMD = areal bone mineral density; BMC = bone mineral content; GWG = gestational weight gain; scBMC = size-corrected bone mineral content; UN/NW = under/normal weight women; OW/OB = overweight/obese women.

partially explain a positive association with offspring bone mass restricted to the low/normal BMI group. Also, under/normal weight women gain more fat than obese women, causing increased nutrient availability and greater exposure to hormones such as leptin and estrogen that may explain a clearer benefit of GWG on offspring bone.¹⁹ Finally, we should not rule out the possibility that the absence of GWG-offspring bone associations in overweight women is attributable to a ceiling effect for bone mass change in response to weight gain due to the attainment of a weight level above which GWG no longer has an effect on intrauterine bone mineral accumulation.

Two artifactual explanations for our findings should also be considered. First, the heterogeneous effect of GWG on the offspring's bone by maternal BMI may have resulted from different GWG distributions. In comparison to overweight

women, mean GWG in under/normal weight women was higher, which may account for the positive GWG-offspring bone associations found in the latter group. However, we observed that heterogeneity by early pregnancy BMI remained similar throughout a wide range of GWG, from -1 kg to 37.5 kg (Supporting Fig. 2). Alternatively, different confounding structures in different strata of maternal BMI could have contributed to the differential associations observed. To rule out this explanation we tested different sets of potential confounders separately in each BMI group—maternal age, height, smoking during pregnancy, gestational diabetes, parity, maternal educational level and employment status, household income, offspring gestational age and sex—and we found that the associations of GWG with bone measures did not change regardless of the confounding structure tested (Supporting Tables 3 and 4).

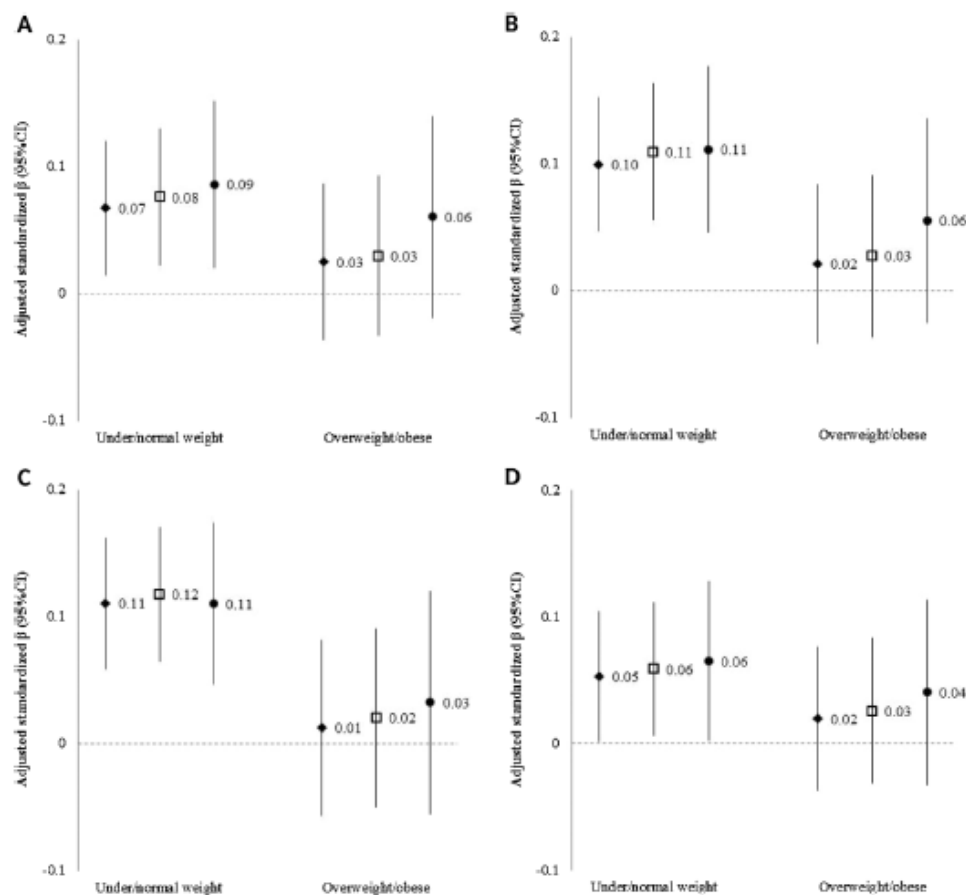


Fig. 3. Adjusted linear regression coefficients (95% CIs) for associations between gestational weight gain and offspring bone measures at 7 years in under/normal weight and overweight/obese women. BMC (A), aBMD (B), scBMC (C), height (D). Values are regression coefficients (95% CIs) that reflect the difference in standardized BMC, aBMD, scBMC, or height per 5-kg increase in gestational weight gain adjusted for maternal age, height and educational level, and gestational age at birth of the offspring. Diamond: whole sample ($n = 1390$ in the under/normal weight group; $n = 775$ in the overweight/obese group); square: only participants with information on self-reported weight in early pregnancy from the primary questionnaire source ($n = 1357$ in the under/normal weight group; $n = 751$ in the overweight/obese group); circle: only participants with lower susceptibility to weight misreporting ($n = 1001$ in the under/normal weight group; $n = 523$ in the overweight/obese group). aBMD = areal bone mineral density; BMC = bone mineral content; GWG = gestational weight gain; scBMC = size-corrected bone mineral content.

We used self-reported weight to compute BMI and GWG. This option might raise some concerns because there is evidence that women underestimate their early pregnancy weight, particularly those who are overweight before pregnancy.^(36,37) To assess the potential impact of reporting bias on our effect estimates, we performed a sensitivity analysis by restricting the sample to women less likely to underreport their weight.^(12,38) GWG-offspring bone associations in women less susceptible to misreporting were essentially similar to those obtained for the whole sample. Accordingly, results from previous studies have shown that reporting error does not significantly change associations between pregnancy-related weight and birth outcomes and that there is high agreement between self-reported and measured pregnancy weights when recall occurs within 1 year after delivery for early pregnancy weight and

within 6 weeks after delivery for predelivery weight,⁽¹²⁾ as was the case in our study.

Although the vast majority of women (94.0%) had BMI and GWG estimated from self-reported weight in early pregnancy we opted to recover information from alternative sources for the remaining 6.0%. Our sensitivity analysis excluding the latter group showed that associations remained similar to those obtained for all participants. These results are consistent with previous evidence about agreement between data sources in Generation XXI, describing small differences between self-reported information and clinical records for pregnancy weight and height.⁽¹³⁾

DXA is a well validated and commonly used technique to assess bone density in children due to speed, precision, safety, low cost, and widespread availability. Because DXA does not provide a measure of true volumetric density,⁽¹⁵⁾ we computed

Table 2. Adjusted Means $\pm$ SD of Z-scored Bone Mass Properties at 7 Years of Age, According to Maternal GWG Categories (Institute of Medicine, 2009), in Under/Normal Weight and Overweight/Obese Women

	Adjusted (mean $\pm$ SD)			Differences means (95% CI)	
	Insufficient GWG	Adequate GWG	Excessive GWG	Adequate – insufficient GWG	Excessive – adequate GWG
BMC Z-score					
Under/normal weight	-0.19 $\pm$ 0.05	-0.09 $\pm$ 0.04	-0.00 $\pm$ 0.05	0.10 (-0.02 to 0.22)	0.09 (-0.03 to 0.21)
Overweight/obese	0.22 $\pm$ 0.09	0.16 $\pm$ 0.06	0.18 $\pm$ 0.05	-0.05 (-0.28 to 0.17)	0.02 (-0.13 to 0.17)
aBMD Z-score					
Under/normal weight	-0.25 $\pm$ 0.05	-0.11 $\pm$ 0.04	0.00 $\pm$ 0.05	0.14 (0.02 to 0.26)	0.12 (-0.00 to 0.24)
Overweight/obese	0.24 $\pm$ 0.10	0.20 $\pm$ 0.06	0.23 $\pm$ 0.05	-0.04 (-0.27 to 0.18)	0.02 (-0.13 to 0.18)
scBMC Z-score					
Under/normal weight	-0.27 $\pm$ 0.05	-0.08 $\pm$ 0.04	0.00 $\pm$ 0.05	0.19 (0.07 to 0.30)	0.08 (-0.04 to 0.20)
Overweight/obese	0.19 $\pm$ 0.11	0.20 $\pm$ 0.07	0.22 $\pm$ 0.05	0.00 (-0.24 to 0.25)	0.02 (-0.15 to 0.19)
Height Z-score					
Under/normal weight	-0.17 $\pm$ 0.05	-0.11 $\pm$ 0.04	0.01 $\pm$ 0.05	0.06 (-0.05 to 0.18)	0.12 (0.00 to 0.24)
Overweight/obese	0.03 $\pm$ 0.09	0.07 $\pm$ 0.06	0.10 $\pm$ 0.04	0.04 (-0.16 to 0.24)	0.03 (-0.11 to 0.17)

Means are adjusted for maternal age, height, educational level, and gestational age at birth of the offspring.
 GWG = gestational weight gain; BMC = bone mineral content; aBMD = areal bone mineral density; scBMC = size-corrected bone mineral content.

and used scBMC as an approximation of volumetric BMD,^(39,40) and we also tested height as an outcome. We did not collect data on in vivo reproducibility to avoid repeated exposure of children to radiation.

This study is strengthened by the use of data from a large prospective population-based birth cohort, assembled during a short period of time, thereby avoiding confounding by age or birth cohort effects. However, we observed differences in maternal characteristics between participants included and the remainder of the cohort that pose a challenge for the generalizability of the findings to the whole cohort. Nevertheless, in our setting the most important correlates of nonparticipation at baseline and attrition throughout follow-up are also determinants of weight misclassification recognized in published literature. Such determinants, including maternal age and educational level, have been considered in our sensitivity analyses to assess the impact of weight misclassification, which was minor. If those findings are extrapolated to the potential impact of attrition and nonparticipation, it seems plausible that our estimates are not substantially biased, which would be compatible with a biological rather than socially-patterned effect of GWG on child bone. A related subject is the generalizability of our findings. Generation XXI is comparatively homogeneous in terms of geographical origin, because only 4.5% of mothers are first-generation immigrants and 3.5% from non-European countries.⁽⁴¹⁾ This background may limit generalizability to other settings, even though our estimates remained essentially unchanged after excluding women born outside the country (data not shown).

In this study we estimated heterogeneous associations between GWG and offspring bone mineralization according to maternal early pregnancy BMI, suggesting a biological interaction between early pregnancy nutritional status and subsequent trajectory. Given the well-known adverse implications of excessive GWG for both mother and offspring on a wide range of outcomes, our findings support that women who are encouraged to follow IOM weight gain recommendations during pregnancy should not expect a deleterious effect on the child's skeletal health.

At the policy level, our findings reinforce IOM guidelines for weight gain. At the clinical practice level, this study supports the inclusion of bone health in the context of weight counseling during pregnancy.

Disclosures

TM, AH, CM, TR, NA, LN, ACS and RL state that they have no conflicts of interest. CC has received personal fees from Alliance for Better Bone Health, Amgen, Eli Lilly, GSK, Medtronic, Merck, Novartis, Pfizer, Roche, Servier, Takeda and UCB, outside of the submitted work.

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"PathMOB: Risco cardiometabólico na infância: desde o início da vida ao fim da infância" (POCI-01-0145-FEDER-016837; Ref. FCT PTDC/DTP-EPI/3306/2014); Unidade de Investigação em Epidemiologia - Instituto de Saúde Pública da Universidade do Porto (EPIUnit) (POCI-01-0145-FEDER-006862; Ref. UID/DTP/04750/2013); Administração Regional de Saúde Norte (Regional Department of Ministry of Health) and Fundação Calouste Gulbenkian; PhD Grant SFRH/BD/92370/2013 (to TM); Postdoc Grant SFRH/BPD/88729/2012 (to RL) co-funded by the FCT and Human Potential Operating Program of the European Social Fund (POPH/FSE Program); FCT Investigator contract IF/01060/2015 (to ACS). This study is also a result of the project DOCnet (NORTE-01-0145-FEDER-000003), supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF). We thank the families enrolled in Generation XXI for their kindness, the members of the research team for their enthusiasm and perseverance, and the participating hospitals and their staff for their help and support.

Authors' roles: Study design: TM, ACS, and RL. Study conduct: TM, AH, ACS, and RL. Data collection: TM and ACS. Data analysis: TM, CO, and RL. Data interpretation: TM, AH, CO, TR, NA, LN, CC, ACS, and RL. Drafting manuscript: TM. Revising manuscript content: AH, CO, TR, NA, LN, CC, ACS, and RL. Approving final version of manuscript: TM, AH, CO, TR, NA, LN, CC, ACS, and RL. RL takes responsibility for the integrity of the data analysis.

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Supplemental data

Brief description of the Generation XXI Birth Cohort

Generation XXI is the first prospective Portuguese population-based birth cohort and aims to characterize prenatal and postnatal growth and development, identifying their determinants in order to understand several aspects of childhood health and to extend follow-up up to adulthood, thereby contributing to health gains among the population.

Recruitment was conducted between April 2005 and August 2006 at the five public maternity units providing obstetrical and neonatal care covering the metropolitan area of Porto, Portugal. All are level III units and, in 2004, were responsible for 91.6% of the deliveries in the whole catchment population, with the remaining occurring in private hospitals/clinics. All mothers who delivered a live-born child with more than 23 weeks of gestation in one of the five units, during the cited period, and resident in the catchment area, were eligible to participate. Seventy percent of the eligible mothers were invited to participate and 91.4% accepted resulting in 8,647 infants enrolled in the cohort study. During the hospital stay, in 24 to 72 hours after delivery, trained interviewers were responsible for the study presentation and subsequent invitation of the mothers.

Descriptive data of baseline characteristics of the original study population is presented in Supplemental table S1.

Four years after birth, between April 2009 and July 2011, a follow-up evaluation was performed and 7459 children, corresponding to 86.3% of the entire cohort, were reevaluated.

Between April 2012 and April 2014, all families were invited once again to attend the 7-years' reevaluation of the cohort and 6,889 children were reassessed (79.7% of the entire cohort).

Similar procedures were adopted throughout all the evaluations. In the face-to-face interviews at baseline and at both follow-ups, information was collected by interviewers, using structured questionnaires concerning the child's health and health of the mother. Data on demographic, socioeconomic and psychosocial circumstances and lifestyles were collected. Personal and family medical history, including gynecologic and obstetric history and prenatal care, was also inquired.

Physical examination of the child including anthropometric, cardiovascular, respiratory and dental evaluation was conducted. An anthropometric evaluation of the mother was performed at baseline and both follow-ups by the interviewers. Blood samples were collected. Routine primary care data regarding the child's development were periodically extracted from the

Child's Health Book. Clinical records at birth and the Pregnancy Booklet were also reviewed to retrieve data on prenatal care, pregnancy complications, delivery and neonatal characteristics. In the 7 years of age follow-up evaluation a consecutive subsample of 3,015 children, corresponding to those children assessed between December 2012 and August 2013 (43.8% of the participants in the 7 years follow-up) was additionally invited to undergo a whole body dual-energy X-ray absorptiometry (DXA). Scanning was successfully performed in 2,408 children (79.9% of the eligible sample and 27.8% of the entire cohort). This additional evaluation took place between March 2013 and February 2014.

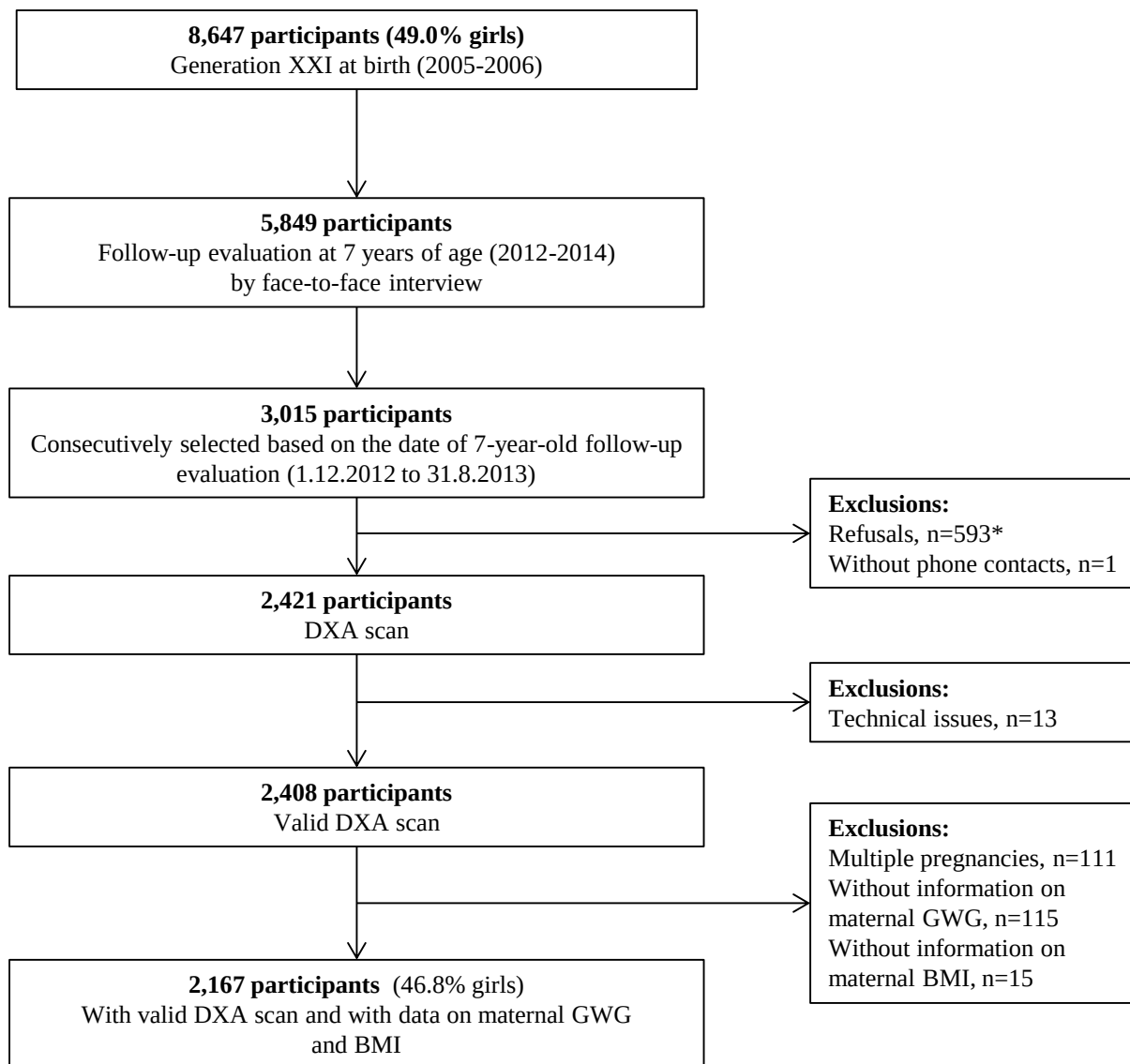
For all the evaluations, interviewers were trained by a multidisciplinary team and were responsible for the application of the questionnaires to the participants, for the physical examination of both family members. DXA scans were performed and evaluated immediately after acquisition by a technician and later validated by a second technician, both with at least 5 years of experience.

Generation XXI study protocol conforms to the ethical principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of the *Hospital de São João* and the University of Porto Medical School and is registered with the Portuguese Authority of Data Protection. Written informed consent was obtained from all parents or legal guardians and oral assent was obtained from children at each evaluation.

Supplemental table S1. Baseline characteristics of Generation XXI participants

Maternal characteristics at birth of child (n=8,495)	
Age, mean (SD), years	29.0 (5.6)
Less than 9 years of formal education (%)	33.4
Unemployment (%)	20.0
Less than 1000 € of monthly household income (%)	40.6
Parity higher than 1 before index pregnancy (%)	10.5
Gestational diabetes in index pregnancy (%)	6.7
Hypertensive disorders of pregnancy in index pregnancy (%)	2.3
First antenatal visit after 12 weeks of gestational age (%)	11.1
Child characteristics (n=8,647)	
Multiples (%)	1.7
Girls (%)	49.0
Gestational age at birth, mean (SD), weeks	38.5 (1.9)
Small for gestational age ^a (%)	14.7
Large for gestational age ^a (%)	4.1

^aSmall and large for gestational age were defined as <10th and >90th percentile, respectively, of sex-specific Kramer growth charts.



Supplemental figure S1. Flowchart of the participants included for analysis, from Generation XXI cohort

Notes: * participants who refused to participate in the DXA evaluation (n=257) + participants who scheduled three different appointments but did not show up for evaluation, or did not respond to our invitation after at least five attempts (n=335) plus one participant with cerebral palsy that did not cooperate
DXA, dual-energy X-ray absorptiometry; GWG, gestational weight gain; BMI, body mass index.

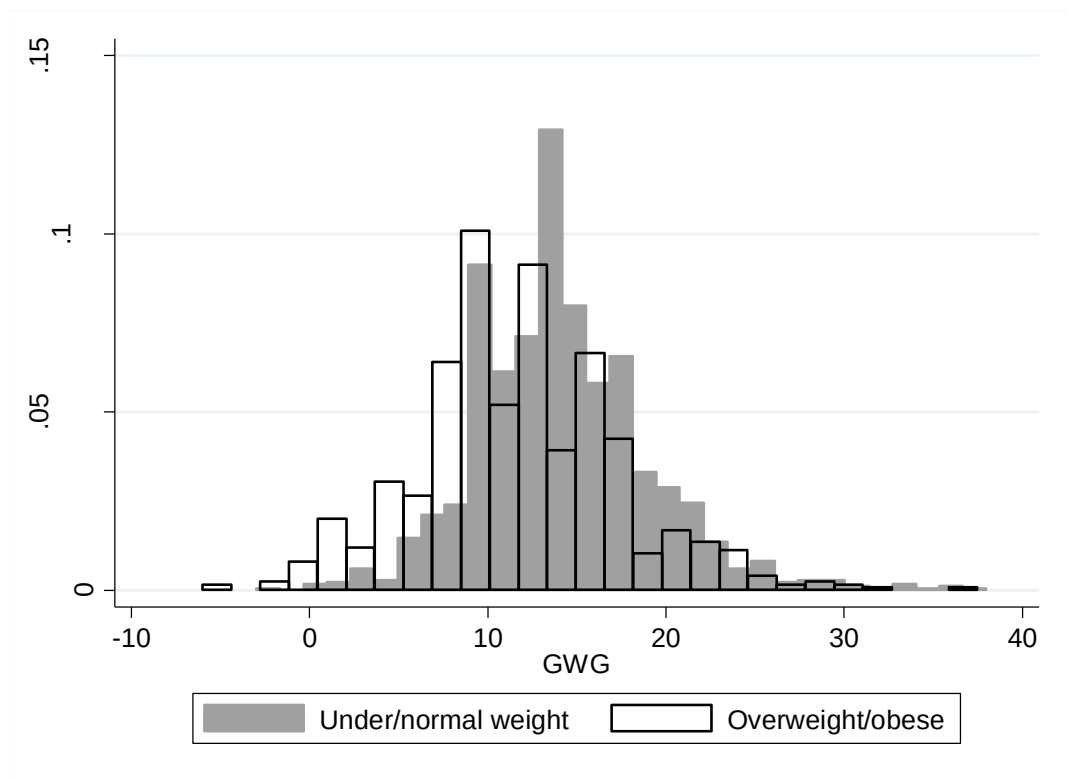
Supplemental table S2. Maternal and child characteristics from included and excluded participants

	Included		Excluded	
Maternal characteristics	n		n	
Age at delivery, mean (SD), years	2,167	29.9 (5.1)	6,322	28.6 (5.8)
No. (%) of women by educational level				
≤9 years		859 (39.7)		3,302 (52.6)
10-12 years	2,165	624 (28.8)	6,274	1,634 (26.0)
>12 years		682 (31.5)		1,338 (21.3)
No. (%) of women by employment status				
Employed		1,711 (79.1)		4,278 (68.6)
Unemployed		336 (15.5)		1,339 (21.5)
Housewife	2,162	71 (3.3)	6,232	422 (6.8)
Student		39 (1.8)		174 (2.8)
Other		5 (0.2)		19 (0.3)
No. (%) of women by monthly household income				
≤1000 €		654 (33.1)		2,313 (43.4)
1001–2000 €		961 (48.6)		2,248 (42.2)
>2000 €	2,119	361 (18.3)	5,995	766 (14.4)
<i>Not known/no answer</i>		143		668
No. (%) of women by previous pregnancies				
None		1,271 (59.1)		3,464 (55.9)
One or more	2,150	879 (40.9)	6,188	2,724 (44.0)
No. (%) of women by early pregnancy BMI groups ^a				
Under/normal weight		1,392 (64.2)		3,994 (66.6)
Overweight/obese	2,167	775 (35.8)	5,998	2,004 (33.4)
Weight gain during pregnancy, mean (SD), kg	2,167	13.2 (5.3)	5,869	12.7 (5.6)
No. (%) of women by smoking status during pregnancy				
Never smoked		1,702 (79.1)		4,716 (75.7)
Ever smoked	2,152	450 (20.9)	6,233	1,517 (24.3)
Child characteristics				
No. (%) of children by sex				
Girl		1,015 (46.8)		3,221 (49.7)
Boy	2,167	1,152 (53.2)	6,480	3,259 (50.3)
Gestational age at birth, mean (SD), weeks	2,167	38.7 (1.6)	6,459	38.4 (2.0)
Birth weight, mean (SD), g	2,167	3,197.6 (475.8)	6,479	3,132.3 (556.6)
Age at 7 years follow-up evaluation, mean (SD), months	2,167	85.2 (2.5)	3,682	85.9 (3.1)
Weight at 7 years, mean (SD), kg	2,165	26.0 (5.0)	3,669	26.4 (5.4)
BMI at 7 years, mean (SD), kg/m ²	2,164	16.9 (2.4)	3,669	17.1 (2.6)

Notes: ^a According to the standard World Health Organization definition

BMI, body mass index.

Results for artefactual explanations for the findings



Supplemental figure S2. Gestational weight gain distribution in under/normal weight and overweight/obese women showing substantial overlap of values in the two groups

Supplemental table S3. Linear regression coefficients (95% CI) for associations between potential confounders and the exposure, i.e. maternal gestational weight gain, in under/normal weight and overweight/obese women, suggesting no different associations between potential confounders and gestational weight gain by maternal BMI group

Potential confounders	Under/normal weight	Overweight/obese
Maternal age at delivery, years	8.01 (-4.86, 20.87)	-4.57 (-23.61, 14.47)
Maternal height, cm	0.11 (0.07, 0.16)	0.05 (-0.01, 0.11)
Maternal educational level		
>12 years	<i>Ref.</i>	<i>Ref.</i>
10-12 years	0.79 (0.15, 1.44)	0.53 (-0.58, 1.64)
≤9 years	0.74 (0.14, 1.34)	-0.70 (-1.73, 0.33)
Employment status		
Employed	<i>Ref.</i>	<i>Ref.</i>
Unemployed	0.73 (0.00, 1.46)	0.25 (-0.81, 1.32)
Housewife/student/other	1.16 (-0.08, 2.39)	-0.90 (-2.51, 0.70)
Monthly household income		
>2000 €	<i>Ref.</i>	<i>Ref.</i>
1001–2000 €	0.10 (-0.59, 0.79)	-0.59 (-1.88, 0.70)
≤1000 €	0.81 (0.05, 1.56)	-0.98 (-2.30, 0.33)
Previous pregnancies		
None	<i>Ref.</i>	<i>Ref.</i>
One or more	-0.42 (-0.95, 0.11)	-2.29 (-3.07, -1.50)
Smoking status during pregnancy		
Never smoked	<i>Ref.</i>	<i>Ref.</i>
Ever smoked	1.44 (0.84, 2.06)	2.43 (1.39, 3.46)
Gestational diabetes		
No	<i>Ref.</i>	<i>Ref.</i>
Yes	-1.46 (-2.72, -0.20)	-1.40 (-2.75, -0.04)
Offspring gestational age at birth, weeks	0.67 (0.52, 0.83)	0.43 (0.19, 0.67)
Offspring sex		
Girl	<i>Ref.</i>	<i>Ref.</i>
Boy	-0.24 (-0.76, 0.27)	0.30 (-0.50, 1.10)

Supplemental table S4. Linear regression coefficients (95% CI) for associations between potential confounders and the outcomes, i.e. offspring bone mass properties at 7 years of age, in under/normal weight and overweight/obese women, suggesting no different associations between potential confounders and bone measures by maternal BMI group

Under/normal weight				Overweight/obese		
Potential confounders	z-BMC	z-aBMD	z-scBMC	z-BMC	z-aBMD	z-scBMC
Maternal age at delivery, years	-0.01 (-0.02, 0.00)	-0.01 (-0.02, -0.00)	-0.01 (-0.02, -0.00)	-0.01 (-0.02, 0.00)	-0.02 (-0.03, -0.00)	-0.03 (-0.04, -0.01)
Maternal height, cm	0.04 (0.04, 0.05)	0.04 (0.03, 0.04)	0.00 (-0.00, 0.01)	0.05 (0.04, 0.06)	0.04 (0.03, 0.05)	0.02 (0.00, 0.03)
Maternal educational level						
>12 years	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>
10-12 years	0.07 (-0.05, 0.20)	0.08 (-0.04, 0.21)	0.07 (-0.05, 0.19)	0.19 (-0.01, 0.39)	0.22 (0.02, 0.42)	0.20 (-0.02, 0.41)
≤9 years	0.07 (-0.05, 0.19)	0.12 (-0.00, 0.24)	0.17 (0.06, 0.28)	0.12 (-0.06, 0.31)	0.17 (-0.02, 0.36)	0.19 (-0.01, 0.39)
Employment status						
Employed	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>
Unemployed	0.07 (-0.08, 0.22)	0.03 (-0.11, 0.18)	-0.08 (-0.22, 0.06)	0.01 (-0.18, 0.20)	0.04 (-0.15, 0.23)	0.07 (-0.13, 0.28)
Housewife/student/other	0.18 (-0.07, 0.43)	0.12 (-0.12, 0.37)	-0.02 (-0.26, 0.21)	-0.12 (-0.41, 0.17)	-0.08 (-0.36, 0.21)	0.00 (-0.30, 0.32)
Monthly household income						
>2000 €	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>
1001–2000 €	0.04 (-0.10, 0.18)	0.06 (-0.07, 0.20)	0.09 (-0.04, 0.22)	-0.18 (-0.42, 0.05)	-0.13 (-0.37, 0.10)	-0.02 (-0.28, 0.24)
≤1000 €	0.07 (-0.08, 0.22)	0.10 (-0.05, 0.26)	0.12 (-0.02, 0.26)	-0.15 (-0.39, 0.09)	-0.10 (-0.34, 0.14)	-0.03 (-0.28, 0.22)
Previous pregnancies						
None	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>
One or more	-0.13 (-0.24, -0.02)	-0.14 (-0.25, -0.04)	-0.10 (-0.20, 0.00)	-0.05 (-0.19, 0.09)	-0.08 (-0.23, 0.06)	-0.14 (-0.30, 0.01)

Smoking status during pregnancy		<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>
	Never smoked	-0.00 (-0.13, 0.12)	0.05 (-0.07, 0.18)	0.14 (0.02, 0.26)	0.02 (-0.17, 0.21)	0.09 (-0.10, 0.28)	0.21 (0.01, 0.41)
	Ever smoked						
Gestational diabetes							
	No	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>
	Yes	0.08 (-0.17, 0.34)	0.12 (-0.14, 0.36)	0.15 (-0.09, 0.39)	0.06 (-0.19, 0.30)	0.14 (-0.11, 0.38)	0.21 (-0.05, 0.47)
Offspring gestational age at birth, weeks		0.01 (-0.02, 0.05)	0.01 (-0.02, 0.05)	-0.00 (-0.03, 0.03)	-0.01 (-0.06, 0.03)	-0.01 (-0.05, 0.04)	0.01 (-0.04, 0.05)
Offspring sex							
	Girl	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>	<i>Ref.</i>
	Boy	-0.00 (-0.10, 0.10)	-0.00 (-0.10, 0.10)	0.00 (-0.09, 0.10)	0.01 (-0.13, 0.16)	0.02 (-0.12, 0.16)	0.01 (-0.14, 0.16)

aBMD, areal bone mineral density; BMC, bone mineral content; GWG, gestational weight gain; scBMC, size-corrected bone mineral content.

4.3. Early Childhood as a Sensitive Period for the Effect of Growth on Childhood Bone Mass: Evidence from the Generation XXI Birth Cohort

Teresa Monjardino, Joana Amaro, Maria João Fonseca, Teresa Rodrigues, Ana Cristina Santos, Raquel Lucas

Abstract

Background: To identify sensitive periods for the effect of early life growth on childhood bone mass we compared the associations between weight and length/height velocities from birth to age six and bone mineral content (BMC) at 7 years of age.

Methods: We analyzed data from 1853 participants from the Generation XXI birth cohort scanned with a whole body dual-energy X-ray absorptiometry system. Velocities of growth in weight and length/height were obtained through linear spline multilevel models on the basis of data collected during routine health examinations. Using linear regression we computed associations of birth weight, birth length, five weight velocities (“early neonatal”: 0-10 days, “early infancy”: 10 days-3 months, “late infancy”: 3-12 months, “early childhood”: 1-3 years, and “later childhood”: 3-6 years) and four length/height velocities (“early infancy”: 0-3 months, “late infancy”: 3-12 months, “early childhood”: 1-3 years, and “later childhood”: 3-6 years) with outcomes BMC, height and height-adjusted BMC at age seven. Confounding by maternal and child characteristics was addressed and effects of growth velocities were adjusted to preceding growth.

Results: Weight and length/height velocities up to the age of six were associated with increased bone mass and height at 7 years with the strongest associations observed for growth in early childhood. In this age period, after concurrent height and confounder adjustment, one standard deviation (SD) increase in weight velocity was associated with higher BMC z-scores: 0.27 (95% CI: 0.22, 0.32) in girls and 0.24 (95% CI: 0.19, 0.29) in boys. Height velocity was also associated with greater height-adjusted BMC z-score: 0.12 (95% CI: 0.07, 0.17) per SD in girls and 0.11 (95% CI: 0.06, 0.16) in boys. The pattern of associations was similar, albeit attenuated, after adjusting for preceding growth.

Conclusion: Growth in second and third years of life may represent a sensitive period for the effect of growth on childhood bone mass, partly through their effect on concurrent body size.

Introduction

Body size, as measured by height and weight, is amongst the strongest determinants of bone mineral properties throughout life, largely reflecting skeletal adaptation to loading, as well as the close constitutional relation between anthropometrics and body composition [1, 2]. Beyond its short-term mechanical effect, there is growing evidence that the velocity of growth in height and weight during infancy and childhood is a probable determinant of later bone mass and density [3-5]. In addition, our previous work has shown that the overall trajectory of weight gain from birth is likely to contribute to cumulative bone mass accrued before puberty, since children in above-average weight gain trajectories had higher bone mineral content and areal density at age seven than those who gained less weight, with the strongest associations for children with persistent weight gain since birth [6]. Nevertheless, from an etiologic perspective, there is an increasing interest in the identification of sensitive periods, during which programmed phenotypes such as bone mass may be particularly responsive to growth velocity [7, 8]. This may provide insights into the timing in childhood when targeted interventions to promote bone health are most promising.

Previous studies of two interval cohorts that examined the effect of specific growth periods on childhood bone mass have found that growth in the first three years of life has the strongest associations with bone properties, with the magnitude of associations decreasing thereafter [9-12]. Despite adding valuable evidence, interval cohorts are by definition examined as part of periodic follow-up assessments whose timing is defined a priori by researchers and whose frequency is necessarily lower than in the context of health care provision. Height and weight records obtained as part of routine health care are a valuable source of information collected at short time intervals that may be used to complement interval cohort data in order to refine the identification of meaningful periods of growth [13]. Specifically, we believe that examining growth velocity during time periods defined empirically on the basis of a large amount of observed data may improve precision in the identification of sensitive periods for the effect of height and weight changes on bone mass measured using dual-energy X-ray absorptiometry (DXA).

Therefore, our objective was to compare, across different age periods defined on the basis of empirical growth data, the associations of height and weight velocities with DXA-derived bone mass at 7 years of age in a large birth cohort.

Materials and Methods

The Generation XXI cohort

All pregnant women delivering live-born infants between April 2005 and August 2006 at each of the five level III maternity units covering the metropolitan area of Porto were invited to participate. Overall, 8647 infants with gestational age above 23 weeks and their mothers were enrolled (91.4% participation). These children were followed up at ages 4, 7 and 10 years. The 13 years evaluation is ongoing as of May 2019. At ages four and seven, 7459 (86.3%) and 6889 (79.7%) children, respectively, had follow-up data. The present study is based on a subsample of 1853 of those children, whose selection criteria and comparison with the remaining cohort participants are described below. The Ethics Committee of *Hospital de São João* approved the study protocol. The study complies with the Ethical Principles expressed in the Helsinki Declaration and with the national legislation and is registered with the Portuguese Authority for Data Protection. In all evaluations, participants were informed about the potential discomfort caused by participation and only children providing oral assent and whose legal guardians provided written informed consent were considered participants. Detailed descriptions of the Generation XXI cohort have been published elsewhere [14, 15].

Growth trajectories

Birth weight and length were obtained from clinical records held at the maternity units and subsequent weight and length/height measurements were abstracted from the National Health Service official children's health books. At the 4 years follow-up evaluation, legal guardians were asked to provide, whenever possible, all measures of the child's weight and length/height from books, comprising data from birth up to 70 months of age, which allowed to model growth up to the age of 6 years. Average weight and length/height growth trajectories were then estimated with linear spline multilevel models. Full details on the modelling of growth trajectories have been described elsewhere [13]. Briefly, the best-fitting splines for length/height and weight, in both girls and boys, had knots at 3 months, 12 months and 3 years, with an extra knot at 10 days for weight. These models were the ones that best fitted observed data and provided interpretable summaries of the pattern of growth throughout childhood. Thus, length/height growth was modelled as four linear splines while weight growth was modelled as five linear splines, characterized by different linear growth rates that are initially higher and then decrease with age (Table 1, from reference [13]). From these models, we obtained individual estimates of weight velocity for five age periods ("early neonatal": 0 to 10 days, "early infancy": 10 days to 3 months, "late infancy": 3 to 12

months, “early childhood”: 1 to 3 years, and “later childhood”: 3 to 6 years) and length/height velocity for four age periods (“early infancy”: 0 to 3 months, “late infancy”: 3 to 12 months, “early childhood”: 1 to 3 years, and “later childhood”: 3 to 6 years). At 7 years of age, height and weight were updated by trained examiners according to standard procedures. Height was measured with a wall stadiometer to the nearest 0.1 cm and weight was measured with a digital scale to the nearest 0.1 kg, while the child was barefoot and in light clothing.

Table 1. Estimated birth weight, length and growth velocities from birth to 6 years predicted by linear spline multilevel models for 5282 children in Generation XXI (Howe LD, et al. (2016) Stat Methods Med Res 25: 1854-1874 [13])

	Girls	Boys	Girls	Boys
	Weight (kg)		Length (cm)	
Birth	3.11 (0.40)	3.22 (0.43)	48.33 (1.87)	48.97 (1.94)
	Weight velocities (kg/month)		Length/height velocities (cm/month)	
Early neonatal	0.42 (0.64)	0.52 (0.70)	3.79 (0.22)	4.12 (0.22)
Early infancy	0.87 (0.16)	0.99 (0.17)		
Late infancy	0.40 (0.08)	0.41 (0.09)	1.70 (0.17)	1.70 (0.18)
Early childhood	0.22 (0.05)	0.22 (0.05)	0.90 (0.07)	0.88 (0.07)
Later childhood	0.19 (0.05)	0.18 (0.05)	0.53 (0.02)	0.51 (0.02)

Values represent mean (standard deviation) of birth weight and length, weight velocities and length/height velocities in girls (n=2611) and boys (n=2671)

Weight velocities for early neonatal (0–10 days), early infancy (10 days–3 months), late infancy (3–12 months), early childhood (1–3 years) and late childhood (3–6 years) periods. Length/height velocities for early infancy (0–3 months), late infancy (3–12 months), early childhood (1–3 years) and late childhood (3–6 years) periods.

DXA-derived bone variables

At the 7 years follow-up, a subsample of children consecutively attending the evaluation (43.8% of the participants) were invited to undergo a whole body DXA. Selection was chronologic and solely related with equipment availability. Scans were performed using a Hologic Discovery QDR® 4500W device (Hologic Inc., Bedford, Massachusetts, USA), software version 13.3.0.1 according to standard manufacturer protocols while children were barefoot in light indoor clothing and without metal accessories. We obtained total body less head bone mineral content (g) (BMC) [16]. Two trained operators checked all scans independently and excluded those showing unacceptable artefacts.

Potential confounders

Maternal and child factors which have been previously associated with early growth and skeletal development through genetic or environmental effects (maternal pre-pregnancy body mass index (BMI), smoking status during pregnancy, age and educational level at childbirth, and child gestational age) were reported in the baseline questionnaire completed within 72 hours after delivery, and complemented with information retrieved from birth records.

Statistical analysis

Analyses presented in this paper were restricted to the 1853 (48.0% girls) participants included in the growth analysis, who had a valid DXA scan and with complete data on all potential confounders. Specifically, of the 7459 participants who attended the 4-year-old follow-up evaluation, 2077 did not provide growth data from health records and 100 were excluded because they were twins, whose birth weight and subsequent growth rates differ considerably from those of singletons. We also excluded 732 participants who did not attend the 7 years evaluation, 2259 who were not invited for bone densitometry on the basis of equipment availability, 377 who refused to perform the DXA scan, 11 whose images had unacceptable technical quality and 50 who did not have complete data for all potential confounders.

Birth weight and birth length, as well as the five weight velocities and the four length/height velocities predicted by the linear spline model, were standardized (z-scored) by sex. Age- and sex-specific z-scores for BMC and height based on the means and standard deviations (SD) derived from the study sample (n=1853) were computed. Race was not collected or taken into account in the calculation of any of the z-scores since the Portuguese Constitution explicitly forbids the collection of statistical data on race, ethnicity or skin color. In terms of potential impact, immigration is comparatively infrequent in the region of Porto compared to other Western European settings or the

USA: only 4.5% of Generation XXI mothers are first-generation migrants, of whom 3.5% from non-European countries [17].

DXA-derived measures of bone mass are strongly influenced by body size, which in turn is a result of overall growth trajectories up to the point of measurement. To address this, we selected the following three outcomes, all measured at age seven: DXA-derived BMC, height, and height-adjusted BMC (through standard multivariate regression adjustment), the latter being an approach to estimate of skeletal growth relative to height up to that point, more closely related to cumulative bone growth than to the physical properties of bone tissue.

Linear regression models were used to quantify associations between each of the exposures (birth weight, birth length, early neonatal weight velocity, early infancy weight/length velocity, late infancy weight/length velocity, early childhood weight/height velocity and later childhood weight/height velocity) and each of the outcomes measured at age 7: BMC, height, and height-adjusted BMC. Regression coefficients represent the change in each standardized outcome per one standard deviation increase in birth weight/length or in weight or length/height velocity in each age period.

We examined the effects of growth on bone outcomes with 4 models. Model 1 represents the crude analysis. Model 2 was adjusted for potential confounders (maternal age, educational level, pre-pregnancy body mass index, smoking during pregnancy and child gestational age). Model 3 was similar to model 2 with further adjustment for earlier growth, i.e. each weight or height velocity was adjusted for birth weight or height, respectively, plus all of the preceding velocities. For weight growth, a model 4 with additional adjustment for birth length, preceding length/height velocities, and length/height velocity over the same age period was fitted to obtain estimates for body weight after accounting for statural growth. Due to the well-documented sex differences in growth and bone accrual since early stages [18, 19], all analyses were stratified by sex. This was an a priori decision, even though when we formally tested for interaction by including cross-product terms with each weight and length/height velocity and sex in the regression models, we found no evidence of statistical interaction.

Sensitivity analyses were conducted to test the robustness of our findings. Owing to possible differences in growth profile, analyses excluding preterm children and children born to women who gained excessive weight during pregnancy, one group at a time, were carried out.

Statistical analyses were carried out with the use of Stata software, version 11.2 for Windows (Stata Corp. LP, College Station, Texas, USA).

Results

Characteristics of the 1853 children and their mothers included in our study are summarized in Table 2. Compared with the remaining cohort participants, children included in our study were similar with regard to maternal prepregnancy BMI, gestational age, birth weight and birth length. Those included were more likely to have older mothers (mean 30.2 years vs 28.6 years) with higher education (32% vs 22%) and employed (79% vs 69%) and less likely to report a monthly household income lower than 1000€ (31% vs 38%). As shown in Table 2, using as reference all Generation XXI participants eligible for growth analysis, mean z-scores for length and weight velocities in the sample included are very close to zero, suggesting that the sample was on average very similar to the remaining participants at each age period. There were no differences between girls and boys in maternal characteristics, gestational age at birth and weight at 7 years. However, when compared to boys, girls were slightly lighter and shorter at birth, shorter at 7 years of age and more likely to have lower DXA-derived bone mass.

Table 2. Maternal and child characteristics: Generation XXI, Porto, Portugal

	Girls (n=889)	Boys (n=964)	<i>p</i> ^a
Maternal characteristics			
Age at childbirth (years), mean (SD)	30.4 (5.1)	30.1 (5.0)	0.135
Educational level (years), n (%)			
≤ 9	364 (40.9)	371 (38.5)	0.425
10-12	239 (26.9)	283 (29.4)	
>12	286 (32.2)	310 (32.2)	
Prepregnancy BMI (kg/m ²), mean (SD)	24.6 (4.7)	24.3 (4.1)	0.186
Ever smoked during pregnancy, n (%)	186 (20.9)	188 (19.5)	0.447
Child characteristics			
Gestational age at birth (weeks), mean (SD)	38.8 (1.6)	38.7 (1.6)	0.232
Preterm birth, n (%)	51 (5.7)	67 (7.0)	0.285
Birth weight, mean (SD)			
Crude (g)	3146.6 (471.6)	3242.4 (480.9)	<0.001
Z-score, WHO reference ^b	-0.24 (1.09)	-0.27 (1.07)	0.560
Birth length, mean (SD)			
Crude (cm)	48.4 (2.0)	49.1 (2.3)	<0.001
Z-score, WHO reference ^b	-0.38 (1.09)	-0.40 (1.22)	0.647
Weight velocity (Z-score ^c), mean (SD)			
Early neonatal	0.04 (0.98)	0.00 (1.05)	0.437
Early infancy	-0.01 (0.98)	0.01 (0.98)	0.745
Late infancy	0.03 (0.96)	-0.02 (0.97)	0.228
Early childhood	-0.01 (1.03)	-0.06 (0.92)	0.327

Later childhood	-0.03 (1.03)	-0.07 (0.92)	0.385
Length/height velocity (Z-score ^c), mean (SD)			
Early infancy	0.03 (0.98)	0.07 (1.02)	0.441
Late infancy	-0.04 (1.02)	-0.00 (0.99)	0.411
Early childhood	-0.06 (1.03)	-0.01 (0.97)	0.219
Later childhood	0.03 (1.07)	0.02 (1.07)	0.831
Weight at 7 years, mean (SD)			
Crude (kg)	27.3 (5.9)	27.0 (5.1)	0.272
Z-score, WHO reference ^b	0.76 (1.13)	0.70 (1.16)	0.220
Height at 7 years, mean (SD)			
Crude (cm)	124.1 (5.5)	125.2 (5.6)	<0.001
Z-score, WHO reference ^b	0.18 (0.91)	0.20 (0.98)	0.634
DXA-derived BMC at 7 years (g), mean (SD)	591.6 (85.4)	600.8 (86.4)	0.021

^a Maternal and child characteristics were compared between girls and boys with two-sample Student's *t* or chi-squared tests.

^b Age and sex-specific Z-scores computed using WHO reference data [18].

^c Sex-specific Z-scores of length/height and weight velocities predicted by the linear spline multilevel models computed based on the means and standard deviations derived from 5282 children from Generation XXI [13].

Age intervals in weight growth: early neonatal (0–10 days), early infancy (10 days–3 months), late infancy (3–12 months), early childhood (1–3 years) and late childhood (3–6 years). Age intervals in length/height growth: early infancy (0–3 months), late infancy (3–12 months), early childhood (1–3 years) and late childhood (3–6 years).

SD, standard deviation; BMI, body mass index; BMC, bone mineral content; DXA, dual-energy X-ray absorptiometry.

Figures 1 and 2 show estimates of the associations between weight and length/height velocities and BMC, height and BMC adjusted for height at 7 years of age, after adjustment for confounders only (model 2) and after additional adjustment for previous growth (model 3). Numerical values of all regression coefficients for models 1 through 4 are presented in Supplemental Tables 1 and 2.

Birth anthropometry

Birth weight was positively associated with bone mass and height in crude and adjusted analysis (Figure 1, models 2 and 4 and Supplemental Table 1, model 1). Birth length was positively associated with BMC and height (Figure 2, model 2 and Supplemental Table 2, model 1). Adjustment for height at 7 years largely attenuated associations between birth anthropometry and BMC (Figures 1 and 2, panels e) and f) and Supplemental Tables 1 and 2).

Weight velocity

Crude and confounder-adjusted estimates revealed a lack of association between early neonatal weight velocity and childhood bone mass (Figure 1, model 2 and Supplemental Table 1, model 1). Additional adjustment for birth weight and length originated negative associations between early neonatal weight velocity and 7-years-old BMC, height and BMC adjusted for height. In contrast, weight velocity in the subsequent four age periods predicted childhood bone mass and height significantly and positively. We found the strongest associations for early childhood weight velocity: after confounder adjustment, 1 SD increase in weight velocity during the second and third years of life was associated with average increases in BMC z-score of 0.57 (95% CI: 0.52, 0.62) in girls and 0.57 (95% CI: 0.51, 0.63) in boys and in height z-score of 0.54 (95% CI: 0.49, 0.60) and 0.57 (95% CI: 0.51, 0.63) (Figure 1, model 2). Adjustment for height measured concurrently to BMC clearly attenuated all associations from early infancy up to later childhood towards the null, even though associations of early childhood growth with BMC persisted: 1 SD increase in early childhood weight velocity was associated with average increases in height-adjusted BMC z-score of 0.27 (95% CI: 0.22, 0.32) in girls and 0.24 (95% CI: 0.19, 0.29) in boys. All estimates were also attenuated upon adjustment for birth weight and preceding weight velocities, but the same overall pattern remained with positive associations for all periods after 10 days of age and greater magnitudes for weight velocity during early childhood (Figure 1, model 3). Additional adjustment for preceding and concurrent velocities of growth in length/height was responsible for further attenuation of most estimates, with the exception of those between later childhood weight velocity and bone and height measures (Supplemental Table 1, model 4). Nevertheless, associations remained strongest for weight velocity during early childhood.

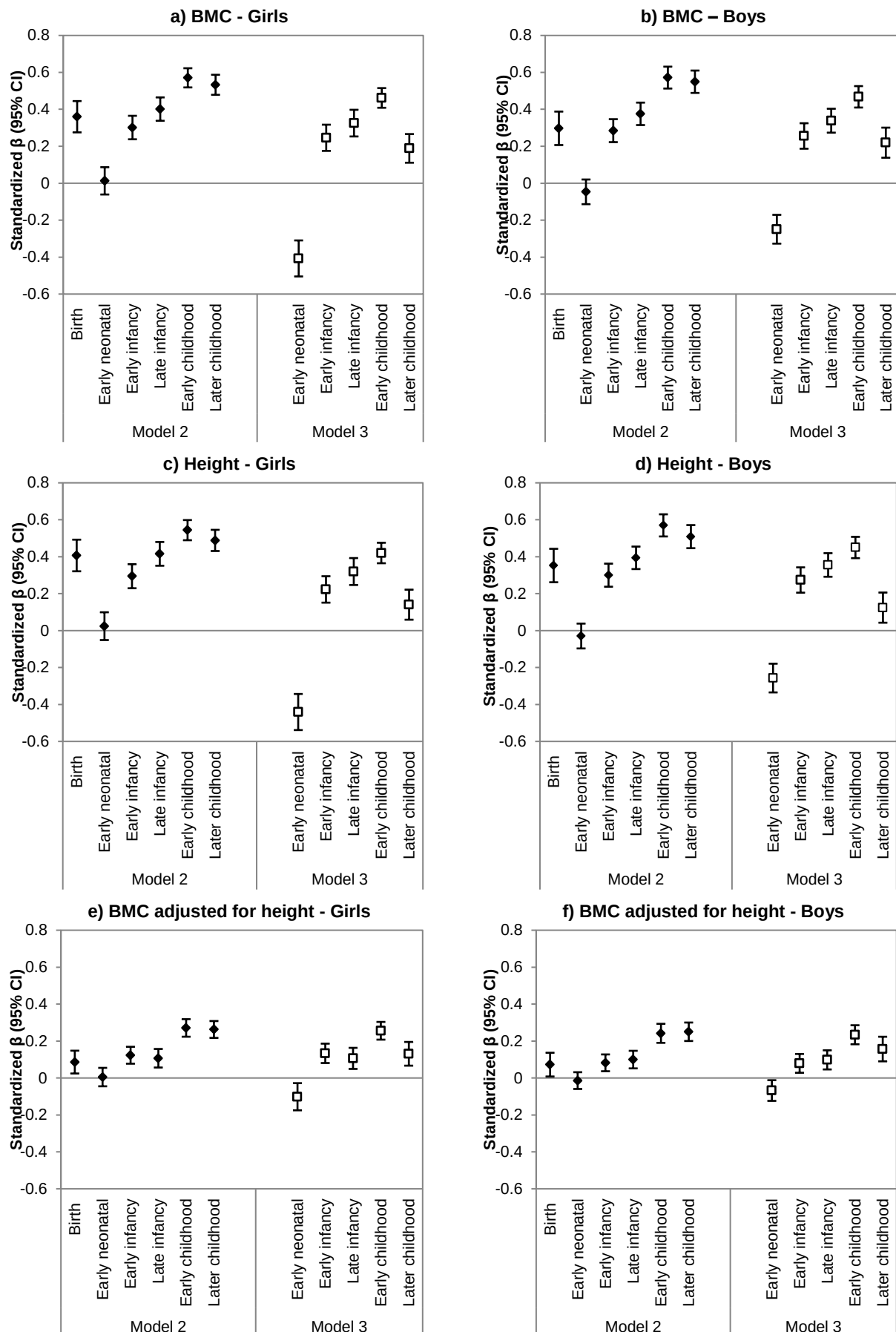


Figure 1. Adjusted linear regression coefficients (95% CI) for the associations between weight velocities and bone mass and height at 7 years of age

Legend: Regression coefficients (95% CI) that represent the change in standardized BMC (a and b), height (c and b) and BMC adjusted for height (e and f) per 1 standard deviation increase in birth weight or in weight velocity in each age period, in girls (left) and boys (right).

Diamond: estimates adjusted for maternal age, educational level, pre-pregnancy body mass index, smoking during pregnancy and child gestational age (model 2). Square: estimates additionally adjusted for birth weight and all of the preceding weight velocities (model 3).

Age intervals: early neonatal (0–10 days), early infancy (10 days–3 months), late infancy (3–12 months), early childhood (1–3 years) and late childhood (3–6 years).

BMC, bone mineral content.

Height velocity

Length/height velocities in early infancy, late infancy and early childhood were positively associated with BMC and height at seven, while later childhood height velocity was only associated with height, with the largest effect estimates being observed during early childhood. In girls and boys, respectively, after confounder adjustment, 1 SD increase in early childhood height velocity was associated with average increases in BMC z-score of 0.45 (95% CI: 0.40, 0.51) and 0.51 (95% CI: 0.45, 0.56) and in height z-score of 0.53 (95% CI: 0.48, 0.59) and 0.64 (95% CI: 0.59, 0.69) (Figure 2, model 2). As observed for weight, adjustment for concurrent height resulted in an attenuation of the estimates but stronger associations for height velocity in early childhood remained: 1 SD increase in height velocity during the second and third years of life was associated with average increases in adjusted for height BMC z-score of 0.12 (95% CI: 0.07, 0.17) in girls and 0.11 (95% CI: 0.06, 0.16) in boys. Also, birth length and preceding length/height velocities adjustment resulted in an attenuation of the estimates (Figure 2, model 3). We found little evidence of association between height velocity in later childhood and bone mass (Figure 2, models 2 and 3 and Supplemental Table 2, model 1).

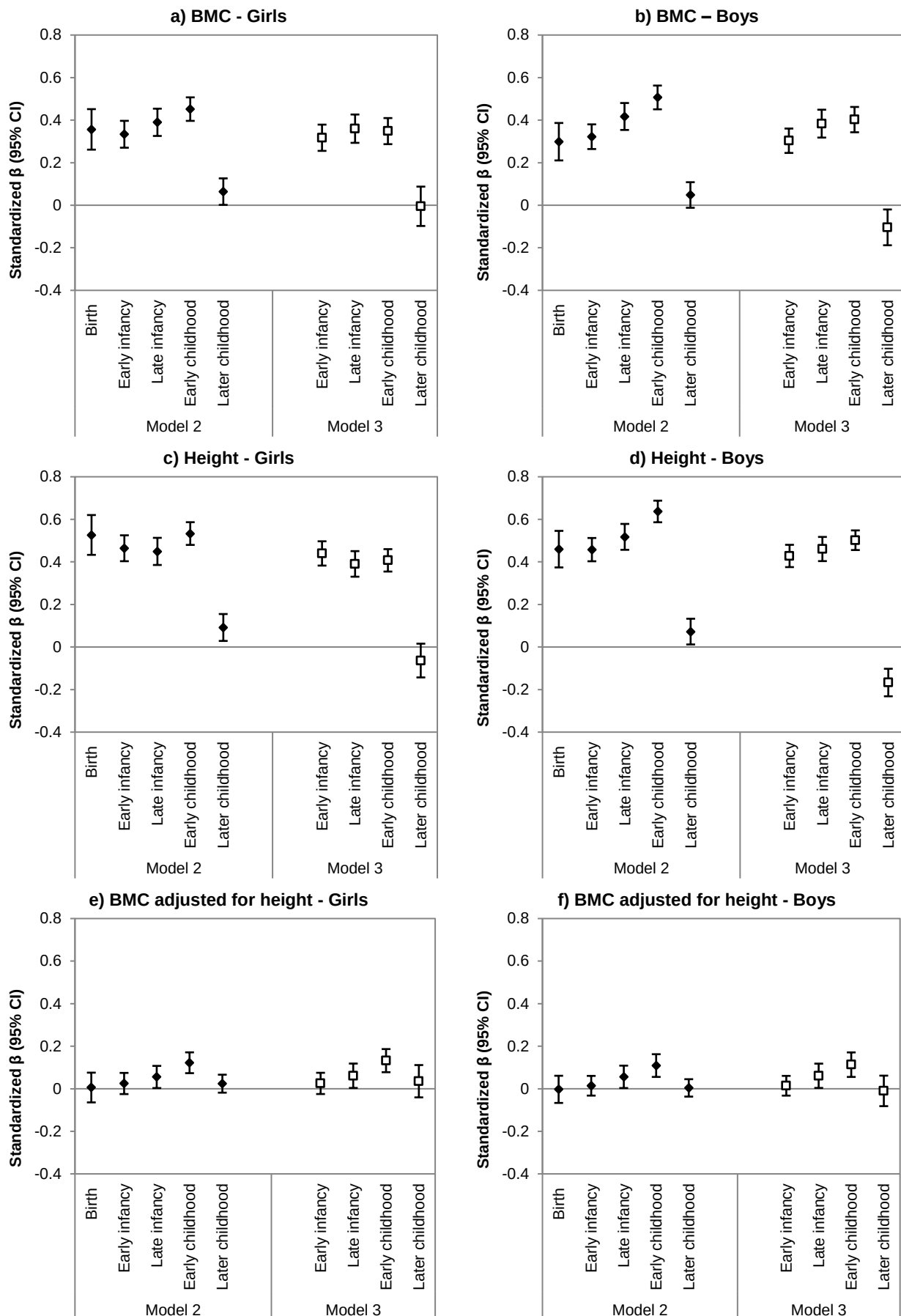


Figure 2. Adjusted linear regression coefficients (95% CI) for the associations between length/height velocities and bone mass and height at 7 years of age

Legend: Regression coefficients (95% CI) that represent the change in standardized BMC (a and b), height (c and d) and BMC adjusted for height (e and f) per 1 standard deviation increase in birth length or in length/height velocity in each age period, in girls (left) and boys (right).

Diamond: estimates adjusted for maternal age, educational level, pre-pregnancy body mass index, smoking during pregnancy and child gestational age (model 2). Square: estimates additionally adjusted for birth length and all of the preceding length/height velocities (model 3).

Age intervals: early infancy (0–3 months), late infancy (3–12 months), early childhood (1–3 years) and late childhood (3–6 years).

BMC, bone mineral content.

Weight vs. height velocities

We found that, in earlier periods, specifically during infancy, length/height velocity was more strongly associated with bone mass at 7 years than weight velocity, while, in early and later childhood, weight velocity stronger correlate with bone mass (Supplemental Table 3). Our results for height were remarkably consistent with those obtained for BMC, which suggest that the relations between growth and bone mass in children are to a large extent attributable to body size. The direction of the associations was also similar for males and females, with only minor differences in the magnitude of the estimates that were not consistently stronger for one gender.

Results from sensitivity analyses after excluding children born preterm, or children born to women who gained excessive weight during pregnancy, showed a similar pattern of associations to those observed for the whole sample.

Discussion

In this population-based birth cohort, height and weight velocity in the first 6 years of life were associated with bone mass at age seven. In comparison to the remaining periods, growth between 1 and 3 years presented the strongest associations with childhood DXA-derived bone mass. Despite a strong statistical dependence on body size, this finding held for both height and weight, and in both genders. Our study suggests that the toddler years might be a particularly sensitive period for the effect of growth on bone development. If a causal interpretation is to be extracted, our results point to the importance of identifying disturbances to normal growth in early childhood that may impact bone health before peak bone mass. From a practical point of view, this study also highlights the informative potential of complementing data from interval cohorts with children's routine health information, with the objective of improving the detection of sensitive periods in life course research.

The generalized assumption that prepubertal bone mass tracks up until peak bone mass and that both determine bone mass into older ages deserves discussion. Studies in several contexts have generally suggested that both body size in the first year of life, as well as growth in height in the first 2 years, are predictive of adolescent and adult bone mass [5, 20-24]. However, evidence of bone mass tracking in longitudinal studies is relatively short-term and its implications are contingent on the assumption of the peak bone mass model [25, 26]. While it is undeniable that childhood is an important period for the attainment of bone strength because of the great amount of bone mineral acquired during this stage, the relevance of early life bone mass acquisition for adult bone strength has been questioned [27, 28]. If bone mass is governed by a homeostatic system acting in the short-term with any perturbation tending to be corrected over time (as per the mechanostat theory), bone mass may depend primarily on recent conditions rather than on the distant past [29]. This work was restricted to estimating an effect of early life growth on later bone mass in prepubertal children and the extent to which such an influence could persist up to peak bone mass and beyond to decrease the risk of fragility fracture is unclear.

DXA-derived BMC measurements are strongly dependent on growth and partially reflect bone size rather than the intrinsic properties of the mineralized tissue. In order to clarify the size dependency of bone mass we used as outcomes not only BMC but also height at age 7, as a measure of linear growth, and BMC adjusted to height, as a measure of bone mass relative to body size. The latter outcome aimed to provide an estimate of

skeletal growth relative to height up to that point, more closely related to cumulative bone growth than to the physical properties of bone tissue. The fact that associations of growth velocities with BMC were strongly attenuated once height was taken into account was expected given the close constitutional relation between bone mass and height. Nevertheless, we acknowledge that the inclusion of current height measurements in the models may represent an adjustment for an intermediate step and could have introduced some degree of collider bias [30].

A number of practical limitations should be noted. First, our study population is composed of children born in Portugal, whose mothers are comparatively homogeneous in terms of geographical origin. In addition, of the overall cohort, 21.4% of children had information on growth and bone variables to be included in the analyses of this study. This proportion is the result of both design choices and attrition or missing data. Children who did not participate were more likely to have lower socioeconomic position than those included in the study, but overall the magnitude of the differences was minor. Also, participants and nonparticipants were similar in terms of the main exposures under study: birth weight, birth length, and growth velocities at each age period. Therefore, we do not expect nonparticipation in Generation XXI follow-up assessments to be strongly related to the study's research question [31]. Another limitation is the fact that growth measures obtained from routine child health records are typically performed by different health professionals with different equipment. Although we were unable to test the validity of routinely-collected length/height measurements in Portugal, this source of information has previously shown little systematic error [32]. Random error is more likely, which would probably reduce the statistical power, although we do not expect this to have had a major impact on our results [33]. Moreover, although we collected detailed information on potential confounding variables, residual confounding due to unmeasured sociodemographic and lifestyle factors could still influence the results.

Despite those potential limitations, our results were consistent with four previous studies that have used regression with measures of conditional growth, residual growth model or two-stage and joint multilevel linear spline models to analyze associations between growth and bone mass and density (aBMD) in children [9, 10, 12]. In a sample of 628 children from the Southampton Women's Survey (SWS), positive associations between growth in height in 1-year time intervals and bone measures at 4 years of age were reported, which were stronger for growth up to 1 year (for BMC) and between 1 and 2 years (for aBMD), than for subsequent growth intervals [9]. Also in SWS, the first and second years of life were identified as the most important age periods for the effect of

conditional growth in height on bone mass at 6 years of age [10]. More recently in SWS participants, stronger associations with 6-year BMC was shown for growth in height between 1 and 3 years (through residual growth models), between birth and 6 months (through two-stage multilevel linear spline models) or between 1 and 3 years (through joint multilevel linear spline models) [11]. Only one study assessed the effect of both height and weight velocities on childhood bone mass and density and suggested different sensitive periods for their effects on aBMD at 6 years of age. In this study from Generation R, weight velocity in the first year of life had the strongest positive association with aBMD, whereas height velocity during the second and third years of life contributed most strongly to aBMD. For BMC, the strongest positive associations were found during the first year of life, for both height and weight velocities [12]. A general observation from previous studies that we also replicate here is a lack of gender heterogeneity in sensitive periods in this life stage.

Our results are also coherent with studies in populations of adolescents and adults that point to the first two years of life, in comparison to subsequent periods, as the most important for the effect of height velocity on BMC/aBMD from 15 to 20 years, on BMC/aBMD in the third decade of life and on BMC at 60 years of age [5, 23, 24]. Our results contradict, however, previous research on the effect of weight velocity on later adolescent and early old age bone mass, where growth closer to the age of bone mass assessment was more strongly associated to DXA-derived bone measures [3, 5]. In another study, weight velocity during childhood was irrelevant as a determinant of bone mass and density at 60 years of age, after entering adult height into the models [24]. Direct comparison with previous research is hampered by differences in the periods of growth identified, which were defined almost always as relative changes in height and weight in interval cohorts, whose timing of measurement is planned in a single chronological age that does not necessarily correspond to the biological age and its intraindividual variation [34].

Positive associations between faster growth in weight or height and childhood bone mass are expected, and likely to be explained by the accretion of both adipose and muscle tissue, which promote osteogenesis both through increased mechanical loading and via the effects of secretory products such as growth hormone (GH), leptin, and cortisol [35]. Therefore, variations in the magnitude of effect estimates between different age periods should be largely attributable to changes in these underlying mechanisms. Our results suggest that the strong association with bone mass of growth in early childhood, in comparison to other early life periods, is related to biological changes specific to the

second and third years of life. A documented underlying change in this life stage includes a change from predominantly GH-independent to GH-dependent growth [36]. In addition to this long-term process, however, we believe that the most likely explanation for the singularity of that period in relation to the remaining stages is the relatively fast acquisition of the vertical posture and the attainment of independent walking at around 12 to 18 months of age, with corresponding changes in gravitational and muscular stimuli to the skeleton [37, 38]. The onset of locomotion seems to be a key macrostructural factor in defining the amount of physical strain imposed on spinopelvic and lower limb bone tissue, as already suggested in our previous work with posture that showed a clear association between sagittal morphotype and bone mass [39]. Changes in this balance of forces are so great from 1 to 3 years that growth in later periods may become comparatively less relevant. From the statistical point of view, this is compatible with later growth being less predictive of bone mass and observable as a decrease in the magnitude of the associations towards later childhood.

As a starting point, we estimated the effects of birth length and weight as markers of prenatal growth. As in previous studies, we found that body size at birth was positively associated with later bone mass, but less strongly than postnatal growth [9, 10, 12, 40]. After birth size adjustment, we found an inverse association between early neonatal weight velocity and childhood bone mass. This is in agreement with our observation that early neonatal weight velocity was inversely related to weight velocities in subsequent age periods (Supplemental Figure 1) which may be due to compensatory growth. Due to national policy, early neonatal measurements are particularly frequent in Portugal, which provides high data density in our sample but scarce opportunities for comparison with other settings.

An additional relevant finding was on the balance between height and weight velocities in terms of their associations with bone mineral content across periods. Length/height velocity was more strongly associated with bone mass than weight velocity in the earlier periods, whereas the relative contribution of weight velocity grew stronger later on in childhood. Indeed, height velocity in later childhood showed weak or negligible (and in one case even inverse) associations with bone mass. This pattern of associations probably reflects linear growth as the main driver of bone accrual in the earlier stages, later surpassed by an increasing importance of changes to bone diameter imposed by weight gain through gravitational and muscle forces [12].

In addition to generally corroborating the body of evidence of the first three years of life as a sensitive period for childhood bone mass, the present study adds important evidence. Specifically, it suggests the strongest role for growth during the toddler years, regarding both height and weight velocities and possibly a major contribution of the upright posture. We believe our analytical approach makes the present results particularly robust since we studied velocities of growth defined from an empirical approach. By taking advantage of a large amount of data, we were able to identify growth periods from statistical knot points that indicate changes in average observed velocities that are likely to have relevant biological meaning. The advantages of linear spline multilevel models to summarize childhood growth have been described in detail [13, 41]. Briefly, this approach models growth data efficiently as it allows using all available data, taking into account different timing and number of measurements between individuals, and not restricting the analysis to those with complete data at all times. In this way, this approach identifies age intervals that differ in velocity of growth, rather than using arbitrary intervals limited by data availability, and deals efficiently with collinearity between repeated measures and bias due to missing data [13, 34]. In Generation XXI, linear spline multilevel models have shown good fit to the observed data. Also, the knot points identified were similar to those in other settings where the same analytical approach was used, which supports the validity of this model to identify periods of growth that are biologically, as well as statistically, relevant [13]. We opted to use DXA, the preferred method to assess bone status in children due to its good accuracy, short analysis time, safety, low cost, and wide availability [42]. Importantly, BMC measured by DXA has been shown to be an accurate predictor of fracture risk, which is ultimately a key clinical validation [43]. We also used measurements of total body less head which is the recommended option for obtaining DXA-derived bone measures in pediatric research, due to precision and reproducibility [16]. In addition, our effect estimates were robust to several sensitivity analyses, as well as to adjustments to maternal and child factors, representing potential confounding by genetic and environmental characteristics, and to adjustments to previous growth.

In this population-based prospective cohort, growth in the first six years of life predicted bone mass at 7 years of age, partly through concurrent body size. Possibly due to the adoption of the upright position and the development of walking abilities, our findings identify the second and third years of life as a potential sensitive period of growth for skeletal development.

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Supplemental Table 1. Linear regression coefficients (95% CI) for the associations between birth weight and weight velocities and bone mineral content and height at 7 years of age

	Girls									Boys								
	BMC			Height			BMC adjusted for height			BMC			Height			BMC adjusted for height		
	β	95% CI		β	95% CI		β	95% CI		β	95% CI		β	95% CI		β	95% CI	
Model 1																		
Birth weight	0.29	0.22	0.36	0.27	0.20	0.35	0.12	0.06	0.17	0.20	0.13	0.27	0.25	0.18	0.32	0.05	0.00	0.09
Early neonatal weight velocity	0.04	-0.03	0.11	0.02	-0.05	0.09	0.05	0.00	0.09	-0.02	-0.08	0.04	0.00	-0.06	0.06	-0.01	-0.05	0.03
Early infancy weight velocity	0.29	0.22	0.35	0.29	0.22	0.35	0.11	0.06	0.15	0.28	0.22	0.34	0.28	0.22	0.35	0.09	0.04	0.13
Late infancy weight velocity	0.40	0.34	0.47	0.41	0.35	0.47	0.11	0.06	0.16	0.38	0.32	0.44	0.39	0.33	0.45	0.11	0.06	0.15
Early childhood weight velocity	0.58	0.53	0.63	0.53	0.47	0.58	0.30	0.25	0.34	0.58	0.52	0.64	0.57	0.51	0.63	0.25	0.20	0.30
Later childhood weight velocity	0.54	0.49	0.60	0.48	0.42	0.53	0.28	0.24	0.33	0.56	0.50	0.62	0.50	0.44	0.56	0.26	0.21	0.31
Model 2																		
Birth weight	0.36	0.28	0.44	0.41	0.32	0.49	0.09	0.02	0.15	0.30	0.21	0.39	0.35	0.26	0.44	0.07	0.01	0.14
Early neonatal weight velocity	0.01	-0.06	0.09	0.02	-0.05	0.10	0.00	-0.04	0.05	-0.05	-0.11	0.02	-0.03	-0.10	0.04	-0.01	-0.06	0.03
Early infancy weight velocity	0.30	0.24	0.37	0.29	0.23	0.36	0.12	0.08	0.17	0.28	0.22	0.35	0.30	0.24	0.36	0.08	0.04	0.13
Late infancy weight velocity	0.40	0.34	0.46	0.42	0.35	0.48	0.11	0.06	0.16	0.38	0.31	0.44	0.39	0.33	0.45	0.10	0.05	0.15
Early childhood weight velocity	0.57	0.52	0.62	0.54	0.49	0.60	0.27	0.22	0.32	0.57	0.51	0.63	0.57	0.51	0.63	0.24	0.19	0.29
Later childhood weight velocity	0.53	0.48	0.59	0.49	0.43	0.55	0.26	0.22	0.31	0.55	0.49	0.61	0.51	0.45	0.57	0.25	0.20	0.30
Model 3																		
Early neonatal weight velocity	-0.41	-0.50	-0.31	-0.44	-0.54	-0.34	-0.10	-0.17	-0.03	-0.25	-0.33	-0.17	-0.26	-0.33	-0.18	-0.07	-0.12	-0.01
Early infancy weight velocity	0.25	0.17	0.32	0.22	0.15	0.29	0.13	0.08	0.19	0.26	0.19	0.32	0.27	0.21	0.34	0.08	0.03	0.13
Late infancy weight velocity	0.33	0.25	0.40	0.32	0.25	0.39	0.11	0.05	0.16	0.34	0.27	0.40	0.36	0.29	0.42	0.10	0.05	0.15
Early childhood weight velocity	0.46	0.41	0.51	0.42	0.36	0.48	0.26	0.21	0.30	0.47	0.41	0.53	0.45	0.39	0.51	0.23	0.18	0.29
Later childhood weight velocity	0.19	0.11	0.27	0.14	0.06	0.22	0.13	0.07	0.20	0.22	0.14	0.30	0.12	0.04	0.21	0.16	0.09	0.22
Model 4																		
Birth weight	0.27	0.15	0.39	0.14	0.02	0.26	0.16	0.07	0.24	0.17	0.05	0.29	0.08	-0.03	0.20	0.12	0.04	0.20
Early neonatal weight velocity	-0.40	-0.50	-0.30	-0.42	-0.52	-0.32	-0.10	-0.18	-0.03	-0.24	-0.32	-0.17	-0.25	-0.32	-0.17	-0.07	-0.12	-0.01
Early infancy weight velocity	0.14	0.06	0.22	0.01	-0.06	0.09	0.17	0.11	0.22	0.15	0.07	0.22	0.07	0.00	0.14	0.10	0.04	0.15
Late infancy weight velocity	0.17	0.09	0.25	0.12	0.05	0.19	0.09	0.03	0.15	0.17	0.09	0.25	0.11	0.04	0.17	0.09	0.03	0.15
Early childhood weight velocity	0.36	0.31	0.42	0.24	0.18	0.29	0.24	0.19	0.29	0.33	0.26	0.39	0.16	0.10	0.21	0.24	0.18	0.30
Later childhood weight velocity	0.19	0.11	0.27	0.14	0.07	0.22	0.14	0.07	0.21	0.27	0.18	0.35	0.18	0.11	0.24	0.18	0.11	0.25

Tables show standardized regression coefficients (95% CI) that represent the change in standardized BMC, height and BMC adjusted for height per 1 standard deviation increase in birth weight or in weight velocity in each age period, in girls and boys.

Model 1: crude analysis. Model 2: adjustment for maternal age, educational level, pre-pregnancy body mass index, smoking during pregnancy and child gestational age. Model 3: Model 2 + adjustment for birth weight and all of the preceding weight velocities. Model 4: Model 3 + adjustment for birth length, all of the preceding and concurrent length/height velocities.

Age intervals: early neonatal (0–10 days), early infancy (10 days–3 months), late infancy (3–12 months), early childhood (1–3 years) and late childhood (3–6 years).

BMC, bone mineral content.

Supplemental Table 2. Linear regression coefficients (95% CI) for the associations between birth length and length/height velocities and bone mineral content and height at 7 years of age

	Girls									Boys								
	BMC			Height			BMC adjusted for height			BMC			Height			BMC adjusted for height		
	β	95% CI		β	95% CI		β	95% CI		β	95% CI		β	95% CI		β	95% CI	
Model 1																		
Birth length	0.27	0.19	0.35	0.35	0.27	0.43	0.06	0.00	0.12	0.19	0.12	0.26	0.30	0.24	0.37	0.00	-0.05	0.04
Early infancy height velocity	0.31	0.25	0.38	0.45	0.39	0.52	0.00	-0.05	0.05	0.32	0.26	0.38	0.45	0.40	0.51	0.01	-0.03	0.06
Late infancy height velocity	0.32	0.26	0.38	0.38	0.33	0.44	0.01	-0.04	0.06	0.35	0.29	0.41	0.42	0.37	0.48	0.05	0.00	0.09
Early childhood height velocity	0.45	0.40	0.51	0.53	0.48	0.59	0.12	0.07	0.17	0.51	0.45	0.57	0.64	0.59	0.69	0.11	0.05	0.16
Later childhood height velocity	0.05	-0.01	0.11	0.09	0.03	0.15	0.00	-0.04	0.05	0.04	-0.02	0.10	0.05	0.00	0.11	0.01	-0.03	0.05
Model 2																		
Birth length	0.36	0.26	0.45	0.53	0.43	0.62	0.01	-0.06	0.08	0.30	0.21	0.39	0.00	0.00	0.00	0.00	-0.07	0.06
Early infancy height velocity	0.33	0.27	0.40	0.46	0.40	0.52	0.02	-0.02	0.07	0.32	0.26	0.38	0.46	0.40	0.51	0.01	-0.03	0.06
Late infancy height velocity	0.39	0.33	0.45	0.45	0.39	0.51	0.06	0.00	0.11	0.42	0.35	0.48	0.52	0.46	0.58	0.06	0.00	0.11
Early childhood height velocity	0.45	0.40	0.51	0.53	0.48	0.59	0.12	0.07	0.17	0.51	0.45	0.56	0.64	0.59	0.69	0.11	0.06	0.16
Later childhood height velocity	0.06	0.00	0.13	0.09	0.03	0.16	0.02	-0.02	0.07	0.05	-0.01	0.11	0.07	0.01	0.13	0.00	-0.04	0.05
Model 3																		
Early infancy height velocity	0.32	0.26	0.38	0.44	0.38	0.50	0.03	-0.02	0.08	0.30	0.25	0.36	0.43	0.38	0.48	0.01	-0.03	0.06
Late infancy height velocity	0.36	0.29	0.43	0.39	0.33	0.45	0.06	0.00	0.12	0.38	0.32	0.45	0.46	0.40	0.52	0.06	0.00	0.12
Early childhood height velocity	0.35	0.29	0.41	0.41	0.35	0.46	0.13	0.08	0.19	0.40	0.34	0.46	0.50	0.46	0.55	0.11	0.06	0.17
Later childhood height velocity	0.00	-0.10	0.09	-0.06	-0.14	0.02	0.04	-0.04	0.11	-0.10	-0.19	-0.02	-0.17	-0.23	-0.10	-0.01	-0.08	0.06

Tables show standardized regression coefficients (95% CI) that represent the change in each standardized BMC, height and BMC adjusted for height per 1 standard deviation increase in birth length or in length/height velocity in each age period, in girls and boys.

Model 1: crude analysis. Model 2: adjustment for maternal age, educational level, pre-pregnancy body mass index, smoking during pregnancy and child gestational age. Model 3: Model 2 + adjustment for birth length and all of the preceding length/height velocities.

Age intervals: early infancy (0–3 months), late infancy (3–12 months), early childhood (1–3 years) and late childhood (3–6 years).

BMC, bone mineral content.

Supplemental Table 3. Linear regression coefficients (95% CI) for the associations of birth weight and birth length (adjusted for each other) and weight and length/height velocities (adjusted for each other) with bone mass at 7 years of age

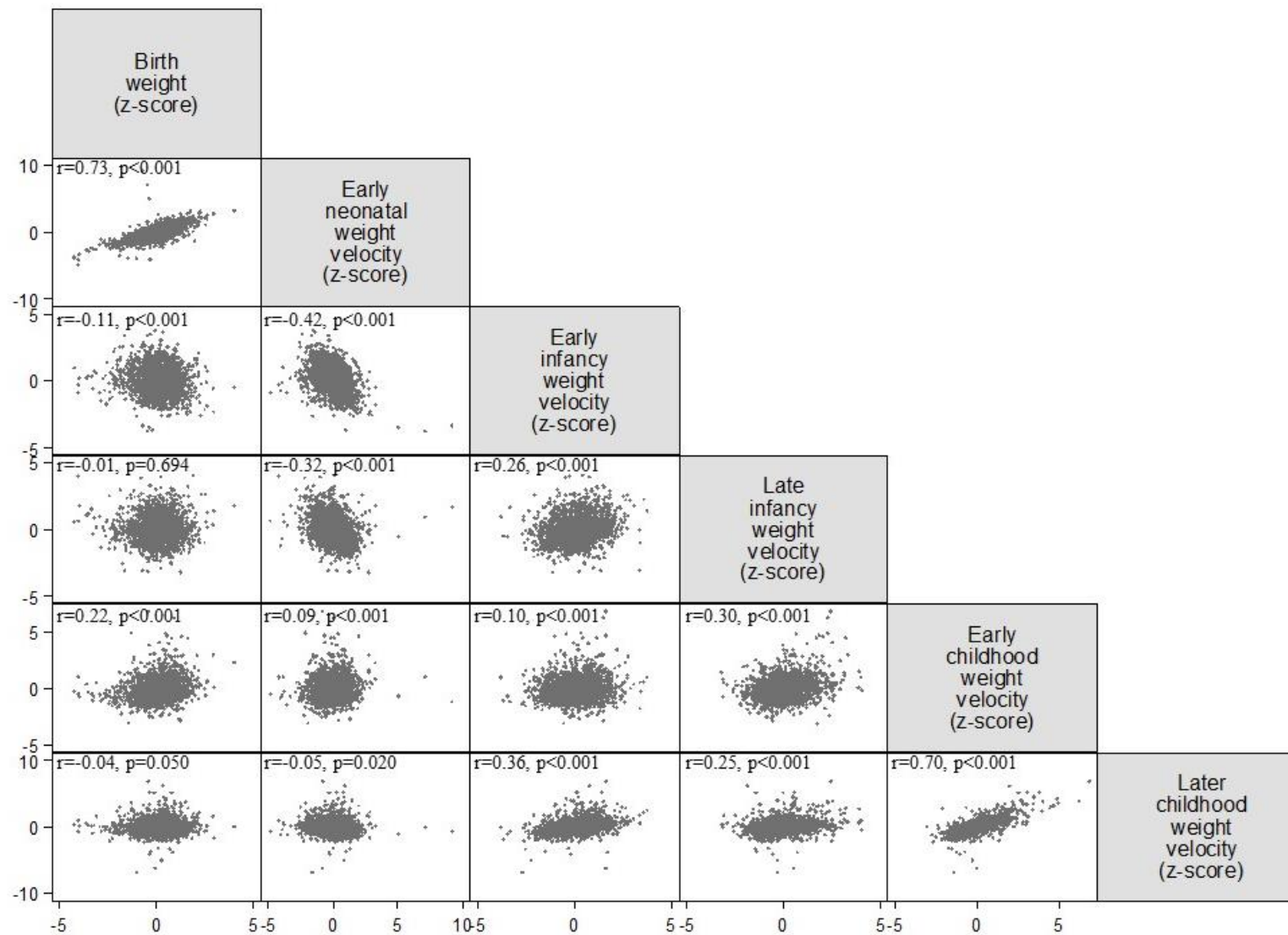
	Girls			Boys		
	β	95% CI		β	95% CI	
Birth weight	0.27	0.15	0.39	0.17	0.05	0.29
Birth length	0.14	0.01	0.28	0.19	0.08	0.31
Early infancy weight velocity	0.17	0.10	0.25	0.15	0.04	4.28
Early infancy length velocity	0.24	0.17	0.31	0.25	0.03	7.36
Late infancy weight velocity	0.27	0.20	0.35	0.21	0.04	5.69
Late infancy length velocity	0.25	0.17	0.32	0.28	0.04	7.08
Early childhood weight velocity	0.45	0.39	0.51	0.38	0.04	10.21
Early childhood height velocity	0.21	0.15	0.27	0.29	0.03	8.41
Later childhood weight velocity	0.55	0.49	0.60	0.59	0.03	18.22
Later childhood height velocity	-0.06	-0.12	-0.01	-0.10	0.03	-3.70

Tables show standardized regression coefficients (95% CI) that represent the change in standardized BMC per 1 standard deviation increase in birth weight/length or in the weight/length/height velocity in each age period, in girls and boys.

In each age period models are adjusted for maternal age, educational level, pre-pregnancy body mass index, smoking during pregnancy and child gestational age.

Age intervals for weight velocities: early infancy (10 days–3 months), late infancy (3–12 months), early childhood (1–3 years) and late childhood (3–6 years). Age intervals for length/height velocities: early infancy (0–3 months), late infancy (3–12 months), early childhood (1–3 years) and late childhood (3–6 years).

BMC, bone mineral content.



Supplemental Figure 1: Pearson correlation coefficients between birth weight and weight velocities in each age period

Age intervals: early neonatal (0–10 days), early infancy (10 days–3 months), late infancy (3–12 months), early childhood (1–3 years) and late childhood (3–6 years).

4.4. Weight Trajectories from Birth and Bone Mineralization at 7 Years of Age

Teresa Monjardino, Teresa Rodrigues, Hazel Inskip, Nicholas Harvey, Cyrus Cooper, Ana Cristina Santos, Raquel Lucas

(J Pediatr. 2017 Dec;191:117-124.e2)



Weight Trajectories from Birth and Bone Mineralization at 7 Years of Age

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Objective To assess whether different trajectories of weight gain since birth influence bone mineral content (BMC) and areal bone mineral density (aBMD) at 7 years of age.

Study design We studied a subsample of 1889 children from the Generation XXI birth cohort who underwent whole-body dual-energy radiograph absorptiometry. Weight trajectories identified through normal mixture modeling for model-based clustering and labeled “normal weight gain,” “weight gain during infancy,” “weight gain during childhood,” and “persistent weight gain” were used. Differences in subtotal BMC, aBMD, and size-corrected BMC (scBMC) at age 7 years according to weight trajectories were estimated through analysis of covariance.

Results Compared with the “normal weight gain” trajectory, children in the remaining trajectories had significantly greater BMC, aBMD, and scBMC at age 7 years, with the strongest associations for “persistent weight gain” (girls [BMC: 674.0 vs 559.8 g, aBMD: 0.677 vs 0.588 g/cm², scBMC: 640.7 vs 577.4 g], boys [BMC: 689.4 vs 580.8 g, aBMD: 0.682 vs 0.611 g/cm², scBMC: 633.0 vs 595.6 g]). After adjustment for current weight, and alternatively for fat and lean mass, children with a “weight gain during childhood” trajectory had greater BMC and aBMD than those with a “normal weight gain” trajectory, although significant differences were restricted to girls (BMC: 601.4 vs 589.2 g, aBMD: 0.618 vs 0.609 g/cm²).

Conclusion Overall, children following a trajectory of persistent weight gain since birth had clearly increased bone mass at 7 years, but weight gain seemed slightly more beneficial when it occurred later rather than on a normal trajectory during the first 7 years of life. (*J Pediatr* 2017;191:117-24).

The theoretical approach currently used to model peak adult bone mass and subsequent fracture risk builds on the premise that there is important tracking of this characteristic throughout the life course, modulated by gene-environment interactions.¹ Empirically, there is also growing evidence of early life influences on bone accrual.^{2,3}

The anthropometric phenotype comprises a set of partially modifiable influences on bone strength from as early as intrauterine life.³⁻⁵ Strong cross-sectional relations between body size and bone mass and density have been established clearly in children, reflecting, to a large extent, the adaptation of skeletal modeling to loading.⁶⁻⁹ More recently, sensitive periods for the effect of height and weight on children's bone mass and geometry have been found,¹⁰⁻¹² suggesting that qualitative differences in the timing of growth may have relevant quantitative impact on bone accrual. However, it is not clear whether differences in the overall shape of growth since birth, particularly weight trajectories, also have differential impact on bone mineral mass and density. Indeed, increased overall exposure to weight during growth, in dose and/or duration, may positively influence bone health as a consequence of greater and/or longer exposure to loading, especially from the lean component.¹³ Among the statistical approaches to examine the role of growth trajectories in later health and disease, group-based modeling methods seek to identify distinctive subgroups of individuals that follow similar growth patterns.^{14,15}

aBMD	Areal bone mineral density
BMC	Bone mineral content
BMI	Body mass index
DXA	Dual-energy radiograph absorptiometry
scBMC	Size-corrected bone mineral content

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So far, few studies have explored the relationship between growth and bone and research has focused on age-delimited periods, rather than capturing the overall shape of weight gain.^{10-12,16-19}

The aim of this study was to estimate the influence of distinct weight trajectories since birth on bone mineral content (BMC) and areal bone mineral density (aBMD) at 7 years of age. Specifically, we aimed to assess whether those trajectories have explanatory power for bone mass at age 7 years in addition to concurrent anthropometrics.

Material and Methods

This work was embedded in the Generation XXI study, a prospective population-based birth cohort established in Porto, Portugal.^{20,21} To summarize, recruitment took place between April 2005 and August 2006 at all level III public units providing obstetric and neonatal care that covered the metropolitan area of Porto. Of the invited mothers, 8495 agreed to participate (91.4%), and a total of 8647 infants (gestational age ≥ 24 weeks) were enrolled. At 4 years of age (April 2009 to July 2011), 7459 children were reevaluated (86.3% of the initial cohort). Again, at 7 years of age (April 2012 to April 2014), 6889 children were reassessed (79.7% of the initial cohort).

The Generation XXI study protocol conforms to the ethical principles outlined in the 1964 Declaration of Helsinki and was approved by the Ethics Committee of Hospital de São João and the University of Porto Medical School and by the

Portuguese Data Protection Authority. Written informed consent from parents (or legal substitute) and oral assent from children were obtained at each evaluation.

In the follow-up evaluation at 7 years of age, children assessed between December 1, 2012, and August 31, 2013, were invited to undergo a whole-body dual-energy radiograph absorptiometry (DXA) scan. From the 5225 children with a weight trajectory assignment, DXA scanning was performed successfully in 1889 children (48.3% girls), who comprised our final sample for analysis (Figure 1). Following standard manufacturer protocols, total body BMC (g), bone area (cm^2), and aBMD (g/cm^2) were obtained while children were barefoot in light clothing and without metal accessories with a Hologic Discovery QDR 4500W device (Hologic Inc, Bedford, Massachusetts), software version 13.3.0.1. To ensure that the lines between adjacent subregions of the body were placed correctly, scans were examined twice, directly after the scanning procedure and at a later time point by a second, well-trained research assistant. We performed daily quality assurance tests using a spine phantom. As recommended by the International Society for Clinical Densitometry for research in pediatric populations, total body less head (subtotal) measures were used.²² Size-corrected bone mineral content (scBMC) was derived separately for girls and boys by linear regression of BMC on bone area and addition of the residuals of the regression to the mean sample BMC.²³

Birth weights were abstracted from clinical records. At ages 4 and 7 years, anthropometric measurements were

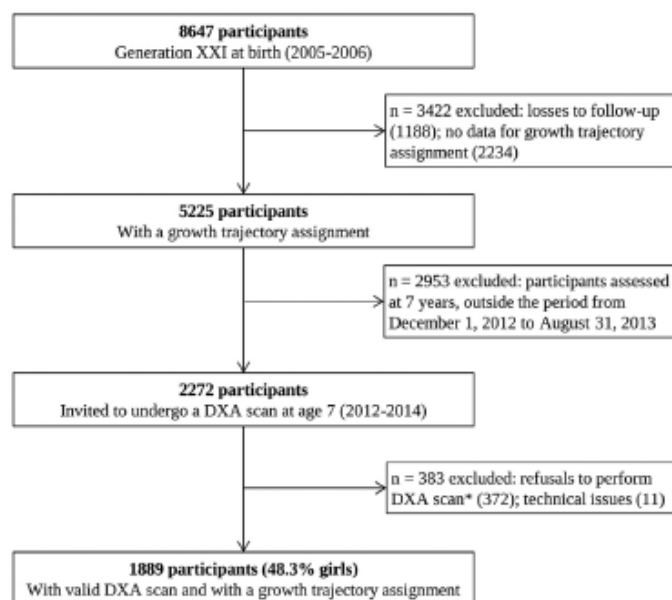


Figure 1. Flowchart of the participants included for analysis from the Generation XXI cohort, Porto, Portugal. *Participants who refused to participate in the DXA evaluation ($n = 171$) + participants who scheduled 3 different appointments but did not show up for evaluation or did not respond to our invitation after at least 5 attempts ($n = 201$).

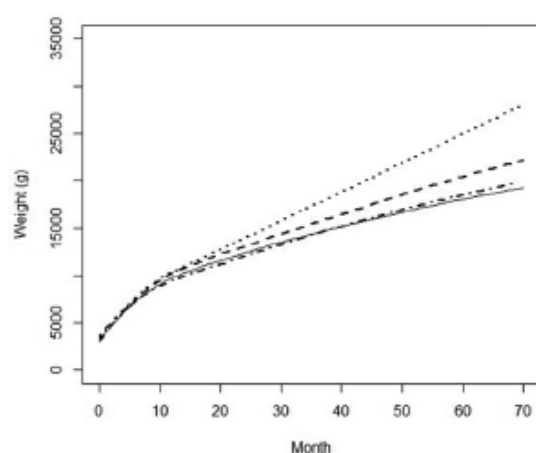


Figure 2. Weight trajectories in the Generation XXI cohort. Solid line, “normal weight gain” trajectory; dashed-dotted line, “weight gain during infancy” trajectory; dashed line, “weight gain during childhood” trajectory; dotted line, “persistent weight gain” trajectory.

performed by trained examiners according to standard procedures. Weight was measured with a digital scale to the nearest 0.1 kg, and height was measured with a wall stadiometer to the nearest 0.1 cm while the child was barefoot and in light clothing. Body mass index (BMI) (kg/m^2) was calculated. At age 7 years, fat mass (kg) and lean mass (kg) were obtained during DXA scanning described for bone assessment. Patterns in weight gain trajectories were identified through normal mixture modeling for model-based clustering in 5225 participants, as previously described.²⁴ Four different weight trajectories for both sexes combined were defined, which are plotted in Figure 2. The 4 trajectories were labeled “normal weight gain,” “weight gain during infancy,” “weight gain during childhood,” and “persistent weight gain.” The “normal weight gain” denomination was chosen because the average weight in this trajectory closely overlapped the 50th weight-for-age percentile curve according to the World Health Organization Child Growth Standards.^{25,26}

To address potential confounding, we selected variables that are plausible common causes of weight trajectories and BMC or aBMD and are not likely to mediate their relation. These included gestational age, maternal background characteristics (age at child birth, prepregnancy BMI, and smoking during pregnancy), and socioeconomic position (measured through maternal educational level at child birth). Data on confounders were self-reported complemented with information retrieved from medical records.

Statistical Analyses

We used 2 approaches to model the effects of weight on childhood bone mineralization: examining individual associations of weight at specific ages and assessing the role of weight

trajectories. First, we computed multiple linear regression of bone BMC, aBMD, and scBMC at 7 years on weight at birth, at 4 and at 7 years of age to estimate regression coefficients, and respective 95% CI. Sex-specific z scores were computed for each DXA-derived bone parameter based on the sex-specific means and SDs derived from the study sample. Age- and sex-specific weight z scores were computed according to the World Health Organization.²⁵ Separate models were fitted for girls and boys. In the second approach, differences in BMC, aBMD, and scBMC according to weight trajectories were estimated through ANCOVA, and adjusted means are presented.

To test the direct effect of trajectories on bone mass not mediated by current body size, adjusted models including height and weight (or fat and lean mass) at 7 years, for BMC and aBMD, or weight (or fat and lean mass), for scBMC, were computed. Post-hoc pairwise comparisons of crude and adjusted means between trajectories were performed with the Tukey-Kramer correction for multiple comparisons. Sensitivity analyses to assess regional effects were carried out by restricting the outcomes to weight-bearing and nonweight-bearing anatomical sites (lower and upper limbs, respectively). Because of growth profile specificities, additional sensitivity analyses were carried out by excluding, consecutively, children born very preterm (gestational age <32 weeks, $n = 11$) and all children born preterm (<37 weeks, $n = 122$). Statistical analysis was performed with Stata, version 11.2, for Windows (Stata Corp LP, College Station, Texas).

Results

Regarding maternal and child characteristics, we observed small differences between participants included in this analysis and all the remaining participants of the cohort (Table I; available at www.jpeds.com). In crude analysis, weight at all ages was associated positively with all DXA-derived bone parameters at age 7 years (Table II). Overall, the magnitude of effect estimates increased with advancing age. After adjustment for height at 7 years, cross-sectional positive associations of weight with BMC and aBMD remained but were strongly attenuated in girls (BMC: standardized $\beta = 0.41$, 95% CI 0.36–0.46; aBMD: $\beta = 0.55$, 95% CI 0.51–0.58) and boys (BMC: $\beta = 0.28$, 95% CI 0.24–0.32; aBMD: $\beta = 0.46$, 95% CI 0.42–0.51). After adjustments for current weight and height, the effect of weight at age 4 years on BMC and aBMD at 7 years also was attenuated strongly but remained positive and statistically significant. Similarly, after adjustment for current weight, birth weight was weakly or not associated with BMC and aBMD. For scBMC, after adjustment for the later weight measure, the direction of the associations with weight at birth and at age 4 reversed (Table II).

In crude analysis, both girls and boys following a “persistent weight gain” trajectory presented the greatest BMC (girls: 674.0 g, boys: 689.4 g) and aBMD means (girls: 0.677 g/cm^2 , boys: 0.682 g/cm^2) followed by children in the “weight gain during childhood” trajectory (BMC [girls: 627.4 g, boys: 646.6 g] and aBMD [girls: 0.635 g/cm^2 , boys: 0.655 g/cm^2]), then those in the “weight gain during infancy” trajectory (BMC [girls: 605.5 g, boys: 631.2 g] and aBMD [girls: 0.621 g/cm^2 , boys: 0.638 g/cm^2]), and lastly by children with a “normal weight gain”

Table II. Associations between weight (at birth, at 4 and at 7 years of age) and BMC, aBMD, and scBMC at 7 years

		Girls				Boys			
		n	BMC z score β (95% CI)	aBMD z score β (95% CI)		scBMC z score β (95% CI)	n	BMC z score β (95% CI)	aBMD z score β (95% CI)
Birth weight z score	912					977			
Model 1		0.21 (0.16-0.27)	0.19 (0.13-0.25)	0.08 (0.03-0.14)		0.13 (0.08-0.19)	0.12 (0.07-0.18)	0.04 (−0.02, 0.10)	
Model 2		0.06 (0.02-0.09)	0.05 (0.01-0.09)	— ^a		−0.00 (−0.04, 0.03)	0.00 (−0.04, 0.05)	— ^a	
Model 3		0.03 (−0.00, 0.06)	0.01 (−0.02, 0.04)	−0.06 (−0.10, −0.02)		−0.00 (−0.04, 0.03)	0.00 (−0.03, 0.03)	−0.03 (−0.08, 0.01)	
Weight at 4 y z score	906					965			
Model 1		0.68 (0.64-0.72)	0.72 (0.69-0.76)	0.54 (0.49-0.59)		0.70 (0.66-0.75)	0.71 (0.66-0.75)	0.40 (0.34-0.46)	
Model 2		0.31 (0.26-0.35)	0.47 (0.42-0.52)	— ^a		0.30 (0.25-0.35)	0.43 (0.37-0.48)	— ^a	
Model 3		0.08 (0.02-0.14)	0.09 (0.03-0.14)	−0.06 (−0.15, 0.01)		0.12 (0.06-0.18)	0.08 (0.02-0.14)	−0.20 (−0.29, −0.12)	
Weight at 7 y z score	912					977			
Model 1		0.66 (0.62-0.70)	0.74 (0.70-0.77)	0.60 (0.56-0.64)		0.61 (0.57-0.65)	0.66 (0.62-0.69)	0.45 (0.40-0.50)	
Model 2		0.41 (0.36-0.46)	0.55 (0.51-0.58)	— ^a		0.28 (0.24-0.32)	0.46 (0.42-0.51)	— ^a	

β , regression coefficient.

The values presented are regression coefficients (SD change in bone outcome per SD change in weight) and 95% CI.

Model 1 represents the crude analysis.

Model 2 is adjusted for height at 7 y of age; *scBMC models were not adjusted for height because height and bone area are extremely correlated.

Model 3 is similar to Model 2 but additionally adjusted for weight at 7 y of age.

trajectory (BMC [girls: 559.8 g, boys: 580.8 g] and aBMD [girls: 0.588 g/cm², boys: 0.611 g/cm²] (Figure 3). Compared with the “normal weight gain” group, bone parameters were significantly greater in all the remaining trajectories. Between participants in trajectories “weight gain during infancy” and “weight gain during childhood,” differences in BMC and aBMD were small and nonsignificant. Regarding scBMC, boys assigned to “normal weight gain” and “weight gain during infancy” did not differ in scBMC (595.6 vs 599.8 g, $P > .05$).

Adjustment of the linear regression coefficients for maternal age, prepregnancy BMI, smoking during pregnancy, socioeconomic position, and gestational age did not change the results appreciably (data not shown). However, after adjustment for height and weight at 7 years of age, associations between weight trajectory and DXA-derived bone parameters were attenuated, and most lost significance. Girls in the “weight gain during childhood” trajectory had the greatest adjusted BMC, which was significantly greater than the estimate for the “normal weight gain” trajectory (601.4 vs 589.2 g, $P < .05$). Also for aBMD, girls with “weight gain during childhood” had the greatest adjusted mean, significantly greater than that in the “normal weight gain” group (0.618 vs 0.609 g/cm², $P < .05$). In boys, increased adjusted mean BMC and aBMD in the “weight gain during childhood” trajectory also was observable, even though differences by trajectories were not statistically significant. For scBMC, the “persistent weight gain” group of girls had significantly greater adjusted mean in comparison with the “normal weight gain” group (600.4 vs 589.8 g, $P < .05$). Boys with a trajectory of “weight gain during infancy” had significantly lower adjusted mean scBMC in comparison with the “normal weight gain” group (593.2 vs 602.1 g, $P < .05$) (Figure 3).

Adjustment for fat and lean mass at 7 years of age, instead of weight, did not change appreciably the associations between weight trajectories and DXA-derived bone parameters. Despite that, lean mass at 7 years was associated more strongly with BMC and aBMD than fat mass. This finding was reversed for scBMC, which was associated more strongly with fat than lean mass (Table III; available at www.jpeds.com).

Sensitivity Analysis

We computed the associations between weight trajectories and bone physical properties at the upper limbs and lower limbs, representing nonweight-bearing and weight-bearing skeletal sites, respectively, and the results of those associations were similar to those found for total body minus head parameters (Table IV; available at www.jpeds.com). Also, after excluding children born very preterm ($n = 11$) and all preterm ($n = 122$) consecutively, associations between weight trajectories and bone parameters remained similar to those found for the whole sample of children.

Discussion

Using data from a large population-based prospective cohort, we found that children who followed a persistent weight gain trajectory had clearly increased bone mass and density at 7 years of age. When differences in concurrent body size were taken into account, later weight gain predicted slightly greater BMC and aBMD than normal weight gain.

Over the conventional approach of studying weight measurements at specific age-delimited periods, longitudinal analysis using mixture modeling has the advantage of classifying overall shapes of growth, whose qualitative differences may have quantitative impact on the outcome assessed.^{14,15,27} The application of trajectory-based models to better understand the etiology and developmental course of health outcomes has grown, but most attention has been devoted to the prediction of cardiometabolic outcomes.^{26,28-33} To the best of our knowledge, this is the first study modeling the effect of overall weight trajectories in relation to bone mineralization.

In both sexes, for crude associations, we found a clear gradient whereby children who remained in a persistent weight gain trajectory had greater bone mass, followed by those who kept intermediate weight trajectories (weight gain during infancy or childhood), and finally by those in a normal weight gain trajectory. This finding is consistent with the observed associations between weight at each age and bone mass and

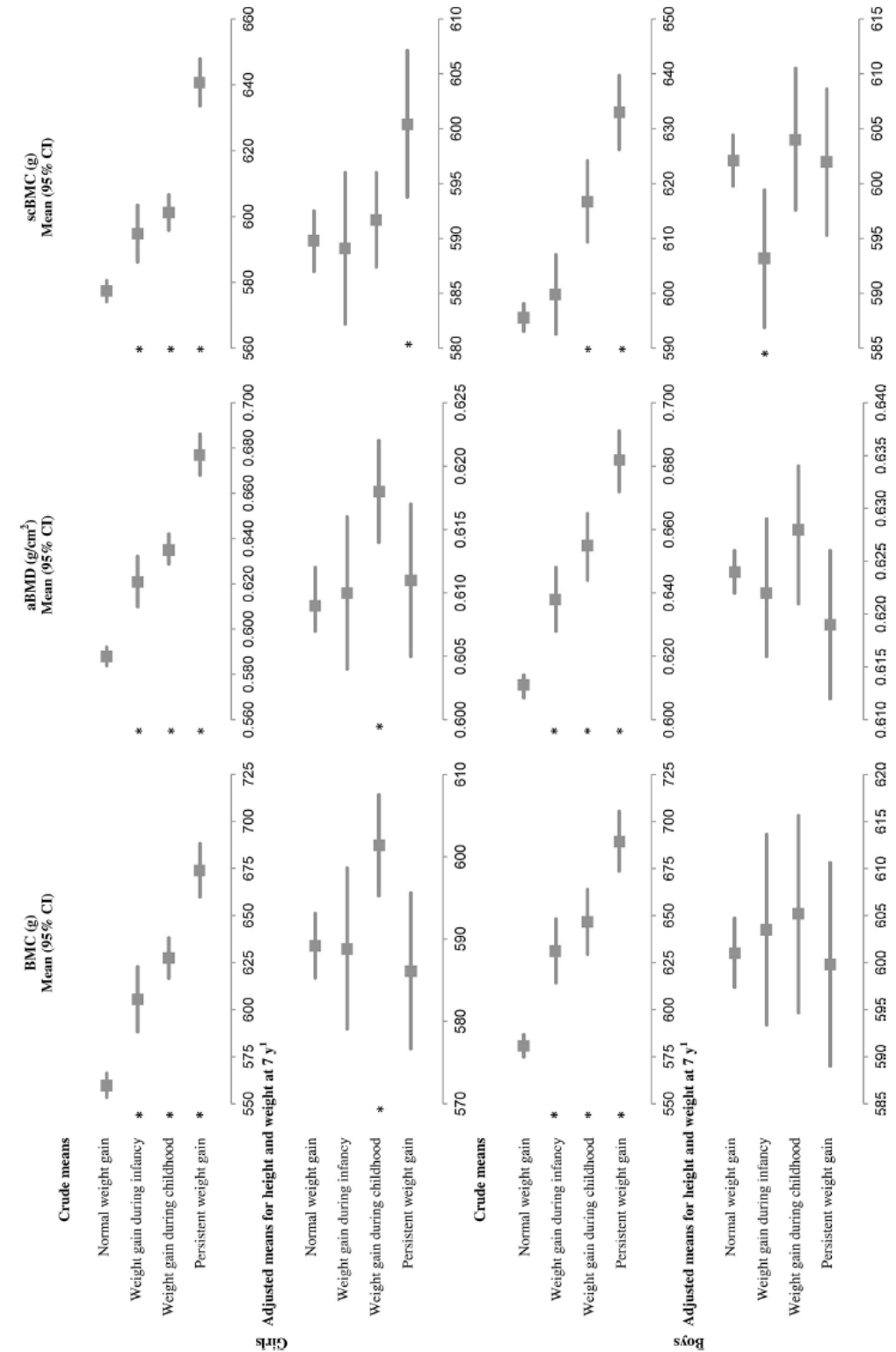


Figure 3. Crude and adjusted means of BMC, aBMD, and scBMC at 7 years of age, according to weight trajectories, and stratified by sex. ¹scBMC means adjusted for weight at 7 years. Asterisks indicate statistically significant differences compared with “normal weight gain” trajectory.

density at 7 years reflecting that long-lasting exposure to loading—both gravitational and muscle loading—is likely osteogenic due to the cumulative effect of enhanced mechanical strain, both in dose and duration. Our results regarding crude associations between weight trajectories and DXA-derived bone parameters were remarkably similar between boys and girls, reinforcing previous evidence that the pediatric skeleton responds positively to increased body weight.³⁴⁻³⁶ Specifically, previous studies have shown strong effects of the mechanical stimulation of body weight on BMC and aBMD from early life and in both sexes.^{19,37,38}

In addition to the total effect of trajectories, we attempted to estimate the direct effect of weight trajectories on BMC and aBMD independent of current body weight. Our finding that those associations were attenuated largely after current weight adjustment is consistent with a strong biological and statistical dependence between the trajectory itself and the final weight attained. If current weight is understood as the result of cumulative growth up to that point (ie, a proxy for the overall trajectory), it becomes both conceptually and technically challenging to disentangle the 2 contributions, and any estimation of the effect of the trajectory is, to a large extent, mediated by current weight.³⁹ In addition, we cannot exclude the possibility that adjustment for current size may have induced collider bias, particularly when trajectory–bone associations changed qualitatively after adjustment.^{39,40} Collider bias refers to the spurious association created between the exposure (weight trajectory) and the outcome (DXA-derived bone parameter), in the model adjusted for the potential mediator (current body size), when there are mediator–outcome confounders,^{41,42} such as unmeasured chronic conditions.

Nevertheless, for a comprehensive interpretation of trajectory–outcome associations, we have presented, as recommended, models with and without adjustment for later size (Figure 3).³⁹ We found relevant differences in the weight-adjusted bone measures that should be highlighted: whereas scBMC remained significantly greater among girls in a trajectory of “persistent weight gain,” we found that children in a trajectory of “weight gain during childhood” had greater BMC and aBMD than the normal weight gain group, after current weight adjustment. We hypothesize that girls and boys in a trajectory of “weight gain during childhood” may represent a group of children with a different metabolic profile that might be of particular significance, as seen in our previous observation with regard to blood pressure.²⁶

With respect to the relative contribution of each component of weight, our results suggest, like previous works, that lean mass is a more important determinant of childhood bone mineralization than fat mass.¹³ Indeed lean mass may be a closer surrogate of both types of mechanical forces acting on the skeleton, muscle, and gravitational, whereas fat mass reflects more directly the influence of gravity.⁴³ This stronger association with lean mass was not observable, however, for scBMC, which possibly represents a distinct dimension from BMC and aBMD.

Previous studies on growth and bone in children have quantified independent effect estimates during age-delimited periods of growth. In the Southampton Women’s Survey, using con-

ditional models of change, heterogeneous effects were observed: growth in the first 2 years of life was associated more strongly with 4- and 6-year-old bone mass and geometry than growth at later ages.^{10,11} In the Generation R study, likewise through conditional growth modeling, growth in height and weight during the first year of life had stronger associations with BMC and BMD at age 6 years than later anthropometrics.¹² Another study in adolescents found, however, that early growth was less associated with bone mass than growth later in childhood.¹⁶ Comparisons between these results and ours are hampered by different methodologic approaches: previous research aimed to identify critical periods of growth for bone development, and we intended to assess the effect of overall growth shapes on bone status.¹⁴

Nevertheless, the results of this study should be interpreted taking into account specific limitations and methodologic options. Bone parameters were assessed by DXA which, despite being well validated and the most commonly used technique to assess bone density in children,²² does not provide a measure of true volumetric density.¹³ Additional limitations of DXA include the systematic overestimation of BMD as bone size increases and the partial artefactual increase of aBMD with weight, due to effect of fat on radiograph absorption. Also, only a single measurement of BMC and aBMD was acquired. Second, the analytical approach used to define weight trajectories shares the limitations of other clustering techniques. These include some degree of subjectivity, given that the process of extracting the number of groups/trajectories is guided by researchers’ decisions, in addition to statistical fit. Because the assignment of individuals to trajectories is based on the highest estimated probability of group membership, latent trajectories should not be interpreted as literally distinct entities but as an approximation to a more complex reality.¹⁵

This study is strengthened by the use of data from a large, prospective, population-based birth cohort with remarkably abundant childhood anthropometric records, which provides greater statistical power than in most previous studies. In addition, by using data from children born during a short period of time, we have minimized the probability of confounding by age or birth cohort effects. Also, the differences in maternal and child characteristics between participants included and excluded were minor in terms of magnitude and should not limit the generalizability of the findings to the overall cohort. In addition, our analysis was conducted with 3 different bone measurements. Even though all measures are 2-dimensional, scBMC is considered an adequate measure of BMC corrected for skeletal size differences and an approximate measure of volumetric BMD.⁴⁴⁻⁴⁶

In this population-based cohort, children following a trajectory of persistent weight gain since birth had clearly increased DXA-derived bone parameters at 7 years of age. Current weight was an accurate proxy of the overall trajectory from birth in terms of its association with bone mass but weight gain seemed slightly more beneficial when it occurred later rather than in an average normal trajectory during the first 7 years of life. This study supports that overall weight trajectory shapes the development of BMC and aBMD, suggesting that changes

to that trajectory, either natural or induced, may modulate bone accrual in childhood. ■

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Table 1. Characteristics of mothers and children included and excluded

Characteristics	Included		Excluded	
	n	Mean (SD) or n (%)	n	Mean (SD) or n (%)
Mother				
Age at delivery, y ^a	1889	30.2 (5.0)	6752	28.6 (5.7)
Prepregnancy BMI, kg/m ² ^a	1868	24.1 (4.2)	6480	23.8 (4.3)
Educational level [†]	1883		6708	
Primary		750 (39.8)		3478 (51.8)
Secondary		533 (28.3)		1767 (26.3)
Higher		600 (31.9)		1463 (21.8)
Smoking during pregnancy [†]				
First trimester	1872	368 (19.7)	6656	1588 (23.9)
Second trimester	1869	224 (12.0)	6650	1108 (16.7)
Third trimester	1869	209 (11.2)	6645	1042 (15.7)
Child				
Sex [†]	1889		6758	
Girls		912 (48.3)		3324 (49.2)
Boys		977 (51.7)		3434 (50.8)
Gestational age, wk ^a	1889	38.7 (1.6)	6737	38.4 (2.0)
Very preterm (<32 wk) [†]		11 (0.6)		112 (1.7)
Moderate/late preterm (32 to <37 wk) [†]		111 (5.9)		592 (8.8)
Age at the 7-y follow-up evaluation, mo ^a	1889	85.1 (2.4)	3953	85.9 (3.1)
Birth weight, g ^a	1889	3194 (481)	6757	3136 (552)
Birth length, cm ^a	1886	49 (2)	6688	49 (3)
Weight at 4 y, kg ^a	1871	17.9 (2.8)	4072	18.3 (3.1)
Height at 4 y, cm ^a	1871	104.9 (4.5)	4063	105.5 (5.3)
Weight at 7 y, kg ^a	1886	25.9 (5.0)	3948	26.4 (5.4)
Height at 7 y, cm ^a	1886	123.4 (5.3)	3948	123.8 (5.4)
Weight trajectories [†]	1889		3336	
Normal weight gain		1250 (66.2)		2013 (60.3)
Weight gain during infancy		158 (8.4)		351 (10.5)
Weight gain during childhood		275 (14.6)		568 (17.0)
Persistent weight gain		206 (10.9)		404 (12.1)

^aMean (SD).[†]n (%).

Table III. Adjusted means of bone physical properties at 7 years of age, according to weight trajectories, and linear regression coefficients (95% CI) for the associations between weight trajectories and bone physical properties (adjusted for fat and lean mass)

	n	BMC			aBMD			scBMC		
		Adjusted means	95% CI	Standardized β	Adjusted means	95% CI	Standardized β	Adjusted means	95% CI	Standardized β
Girls	912									
Normal weight gain		589.5	585.8-593.3	0	0.610	0.607-0.612	0	589.7	586.9-592.5	0
Weight gain during infancy		590.3	580.8-599.6	0.01	0.611	0.605-0.617	0.03	589.1	582.2-596.1	-0.01
Weight gain during childhood		601.2	595.3-607.0	0.14	0.618	0.614-0.622	0.14	591.7	587.4-596.1	0.05
Persistent weight gain		584.2	575.1-593.3	-0.06	0.610	0.604-0.616	0.00	600.7	594.0-607.4	0.26
Fat mass at 7 y z score				0.13		0.08-0.17	0.26			0.44
Lean mass at 7 y z score				0.44		0.37-0.50	0.58			0.27
Boys	977									
Normal weight gain		601.2	597.8-604.7	0	0.624	0.622-0.626	0	602.1	599.8-604.3	0
Weight gain during infancy		603.0	593.4-612.6	0.02	0.622	0.616-0.628	-0.05	593.1	586.8-599.4	-0.25
Weight gain during childhood		606.2	596.3-616.1	0.06	0.629	0.623-0.635	0.08	604.1	597.7-610.6	0.06
Persistent weight gain		598.0	587.7-608.2	-0.04	0.618	0.611-0.624	-0.13	602.1	595.4-608.8	-0.00
Fat mass at 7 y z score				0.08		0.04-0.12	0.20			0.35
Lean mass at 7 y z score				0.49		0.43-0.55	0.70			0.31

The values presented are regression coefficients (SD change in bone outcome per SD change in fat mass and lean mass) and 95% CIs. BMC and aBMD models are adjusted for height.

Table IV. Linear regression coefficients (95% CI) for the associations between weight trajectories and bone physical properties at 7 years of age, in subtotal, weight-bearing, and nonweight-bearing skeletal sites

	Subtotal	BMC z score			aBMD z score			scBMC z score		
		Nonweight-bearing skeletal site (upper limbs)	Weight-bearing skeletal site (lower limbs)	Subtotal	Nonweight-bearing skeletal site (upper limbs)	Weight-bearing skeletal site (lower limbs)	Subtotal	Nonweight-bearing skeletal site (upper limbs)	Weight-bearing skeletal site (lower limbs)	Subtotal
Normal weight gain	0.56 (0.41-0.70)	0.54 (0.39-0.69)	0.52 (0.37-0.67)	0.54 (0.39-0.68)	0.56 (0.41-0.70)	0.52 (0.38-0.67)	0.24 (0.09-0.40)	0.47 (0.32-0.61)	0.52 (0.37-0.67)	0
Weight gain during infancy	0.74 (0.63-0.86)	0.71 (0.60-0.83)	0.72 (0.60-0.83)	0.78 (0.67-0.90)	0.73 (0.62-0.85)	0.77 (0.66-0.88)	0.52 (0.40-0.64)	0.64 (0.53-0.76)	0.72 (0.60-0.83)	0
Weight gain during childhood	1.28 (1.15-1.41)	1.22 (1.09-1.36)	1.22 (1.08-1.35)	1.43 (1.30-1.56)	1.50 (1.38-1.63)	1.45 (1.32-1.58)	1.26 (1.12-1.39)	1.50 (1.37-1.62)	1.22 (1.08-1.35)	0
Persistent weight gain										

4.5. Bone Formation and Resorption Markers at 7 Years of Age: Relations with Growth and Bone Mineralization

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Abstract

Purpose: We aimed to describe remodeling markers in generally healthy prepubertal children using total alkaline phosphatase (tALP), osteocalcin (OC) and β -isomerized C-terminal telopeptides of type I collagen (β -CTX) serum concentrations and to estimate markers' correlations with anthropometric growth (height, weight, body mass index and trajectories of weight gain) as well as bone mineral content (BMC) and areal density (aBMD).

Methods: We assessed 395 7-year-old children from the Generation XXI cohort with tALP, OC and β -CTX concentrations determined from a fasting venous blood sample and BMC/aBMD measured by dual-energy X-ray absorptiometry. Gender-specific reference intervals for tALP, OC and β -CTX in 7-year-old children were established by calculating the 2.5th and 97.5th percentiles. Pearson and partial correlation coefficients (controlling for sex, age, body size and season) between bone markers and growth measures were computed.

Results: tALP increased with height (r_{partial} controlled for sex=0.26, 95% CI: 0.17, 0.35), was higher in overweight than in healthy weight children, and in children who gained weight above average during infancy. No correlations were found between OC or β -CTX and growth. In girls, OC was slightly correlated with subtotal BMC (r_{partial} =0.22, 95% CI: 0.08, 0.35), subtotal aBMD (r_{partial} =0.20, 95% CI: 0.06, 0.33) and lumbar spine aBMD (r_{partial} =0.23, 95% CI: 0.09, 0.36). tALP and β -CTX were not correlated with any of the DXA-derived bone measures.

Conclusion: This study contributed to the description of bone turnover at 7 years of age and suggested that bone metabolism markers measured in a single point in time have limited ability to describe anthropometric growth and bone mass in generally healthy prepubertal children.

Introduction

Serum bone metabolism markers are bone-derived molecules released into circulation as a result of osteoblast or osteoclast activity that can be used to describe bone formation or resorption, respectively [1]. In adults, bone turnover markers reflect the lifelong process of bone remodeling, and have been proposed as independent predictors of bone density and fracture risk [2, 3]. They have also been used to assist the selection of drug treatments for osteoporosis and to monitor the effectiveness of antiresorptive therapies [4, 5].

In children, the meaning of bone metabolism markers is more complex, as they reflect not only background homeostatic remodeling but also two intensive processes that are hallmarks of growth, i.e. bone modeling and linear growth of the skeleton [6]. As such, bone metabolism markers are mainly used in clinical pediatrics for the monitoring of certain chronic conditions that interfere with bone homeostasis and thus with normal skeletal growth and development, including *osteogenesis imperfecta*, juvenile idiopathic arthritis, and chronic kidney disease [7]. Much less is known about the usefulness of bone formation and resorption markers to describe bone development or growth trajectories in the healthy skeleton. From a practical perspective, for instance in large-scale epidemiological studies aiming to assess bone mineralization in population-based samples of children, measurement of serum levels bone formation and resorption could be an interesting alternative to bone mass estimation using dual-energy X-ray absorptiometry (DXA), since the former may demand lower resources, while avoiding radiation exposure [7]. Bone metabolism markers have also the potential to be more sensitive to short-term changes to bone turnover rates [6]. Nevertheless, it is not clear at present whether serum levels of bone metabolism markers add useful information to describe childhood growth, given their high preanalytical variability [8].

Most previous studies exploring the relation between bone metabolism markers and growth have used cross-sectional designs and have described associations in relatively small samples of children, with wide age ranges [9-17]. Additionally, most existing evidence was obtained from convenience or clinical-based samples where distributions of bone markers are not necessarily representative of those in the source population [18, 19]. Therefore, by using data from a population-based sample of prepubertal children of the same age, we aimed to describe bone formation and resorption markers using total alkaline phosphatase (tALP), osteocalcin (OC) and β -isomerized C-terminal telopeptides of type I collagen (β -CTX), and to assess whether those bone metabolism markers are associated with DXA-derived bone properties and anthropometric growth up to seven years of age.

Material and Methods

We used data from a sub-sample of 395 children, recruited and followed up as part of Generation XXI, a prospective birth cohort assembled in 2005/06 at public maternity units in Porto, Portugal [20,21].

Generation XXI cohort assembly and follow-up

All live infants with more than 23 weeks of gestation, born in one of the five public maternity units in the Porto Metropolitan Area, between April 2005 and August 2006, were eligible to participate. Of the invited mothers, 91.4% accepted to participate and their 8647 infants were enrolled in the cohort study. Seven years after birth (April 2012 to April 2014), all Generation XXI participants were invited to a follow-up evaluation and, of the initial cohort, 5849 (67.6%) were interviewed face to face. In each evaluation, we obtained written informed consent from parents or legal guardians and additional oral assent from children at 7 years of age. The study protocol complies with the 1964 Declaration of Helsinki and was approved by the Ethics Committee of *University of Porto Medical School/Hospital de São João*, Porto, Portugal, and registered with the Portuguese Data Protection Authority.

Study sample

From the 5849 children evaluated at 7 years of age we selected a subsample of 400 participants to measure bone metabolism markers and bioactive molecules: serum 25-hydroxyvitamin D (25(OH)D), intact parathyroid hormone (PTH_i), calcium (Ca) and inorganic phosphorus (P_i). Sample size was calculated based on expected correlations between bone parameters, in order to allow estimating a 0.15 linear correlation coefficient with an 85% power at a 0.05 significance level. These participants were selected based on the following characteristics: 1) to be a singleton full-term baby (more than 36 weeks of gestation); 2) to have a valid whole body DXA scan at 7 years, 3) to have a fasting blood sample collected in the morning with enough volume to perform the intended laboratory analyses. From all participants with the aforementioned characteristics, we randomly selected 200 boys and 200 girls. After samples were sent for analysis, we excluded a further five children as their samples were hemolytic, icteric or lipemic, leaving 395 children for the current analysis (49.9% girls).

Children included in the present study were not different from the remaining cohort regarding sex distribution (49.9% girls vs. 49.0%, $p = 0.719$), height at 7 years of age (123.3 vs. 123.7 cm, $p = 0.146$) and weight at 7 years of age (25.9 vs. 26.2 kg, $p = 0.176$).

However, participants included had lower bone mineral content (BMC) (585.0 g in included vs. 604.8 g in excluded participants, $p < 0.001$) than those excluded.

Blood sampling and serum determinations

At the 7 years of age follow-up evaluation, we collected blood samples drawn from an antecubital vein, in the morning after overnight fasting. All children were offered local dermal analgesia with lidocaine/prilocaine (EMLA®). Blood samples were collected in Vacumed® vacuum tubes with gel separator plus clot activator (FL medical, Italy), allowed to clot for 20 minutes at room temperature and centrifuged for 15min at 1500 ×g. Serum was aliquoted and kept frozen at -80 °C in the biobank at the University of Porto Medical School until the day of analysis. All analytical measurements presented in this paper were conducted at the clinical pathology laboratory of the *Centro Hospitalar São João, EPE* in Porto, Portugal, in June 2014, after being thawed according to standard operating procedures.

Total alkaline phosphatase (tALP) was quantified by a spectrophotometric method based on the conversion of p-nitro-phenylphosphate (pNPP) to p-nitrophenol (pNP) measured at 410/480 nm on an Olympus AU5400 analyzer (Beckman Coulter Inc., USA) (the detection range of the kit, defined by the detection limit and the calibration curve maximum concentration, is 5-1500 units/l, U/l).

Osteocalcin (OC), β -isomerized C-terminal telopeptides of type I collagen (β -crosslaps, β -CTX), and parathyroid hormone measurements were performed by electrochemiluminescence immunoassays (ECLIA) in a Cobas e411 analyzer (Roche Diagnostics GmbH, Mannheim, Germany) [22]. The Elecsys N-MID Osteocalcin assay (Roche Cobas, Roche Diagnostics, Mannheim, Germany) detects both the stable N-terminal mid-fragment of OC (amino acids 1–43) and intact Osteocalcin (aminoacids 1–49) (detection range: 0.500-300 μ g/l). The Elecsys β -crosslaps assay/serum quantifies all degradation fragments of the C-terminal telopeptide region of type I collagen α 1 chain that contain the β -isomerized octapeptide EKAHD- β -GGR twice (β -CTX) [23]. This test uses two mouse monoclonal antibodies directed against different regions of the EKAHD- β -GGR octapeptide (detection range: 10-6000 ng/l). Parathyroid hormone was measured as PTHi, a single polypeptide chain containing 84 amino acids. Two different monoclonal antibodies, directed against the aminoacid regions 26-32 (N-terminal) and 37-42 (C-terminal) were used (detection range: 0.127-530 pmol/l).

Serum 25(OH)D concentration was determined using LIAISON® 25-OH Vitamin D Total assay (DiaSorin, Saluggia, Italy), a direct competitive chemiluminescence immunoassay which recognizes 25 (OH) vitamin D2 and 25 (OH) vitamin D3 and is fully automated using a Liason platform (detection range: 4.0-150 ng/ml) [24].

Ca and P_i were determined using the Olympus AU5400 analyzer. Ca was detected by a colorimetric assay where Calcium ions (Ca²⁺) react with Arsenazo III and produce an intense purple color complex (detection range: 1-5 mmol/l) (Beckman Coulter AU System Calcium Arsenazo, Beckman Coulter). P_i was determined by a colorimetric assay where the absorbance at 340/380 nm is directly proportional to the P_i level in the sample (Beckman Coulter AU System Inorganic Phosphorus Reagent, Beckman Coulter) (detection range: 0.32-6.40 mmol/l).

All analytical measurements were performed according to the manufacturer's instructions regarding preventive maintenance, function checks, calibration, and quality control of both tests and equipment. All samples tested underwent automated interference analysis for hemolysis, hyperbilirubinemia and lipemia.

Physical examination

Anthropometry was obtained while the child stood barefoot in light indoor clothing. Weight was measured to the nearest 0.1kg using a digital scale (TANITA, Arlington Heights, IL, USA) and height was measured to the nearest 0.1cm using a wall stadiometer (SECA, Hamburg, Germany). Body mass index (BMI, kg/m²) was calculated as the ratio of weight to height squared and BMI-for-age values were classified according to the World Health Organization reference data for BMI z-score into the following categories: normal weight ($\leq +1$ SD) and overweight ($> +1$ SD, including children with overweight and obesity) [25]. In our sample no children were underweight according to the cut-off value < -2 SD. The definition of weight trajectories in the cohort was based on an extensive body of anthropometric measurements abstracted from the children's health books, recorded in routine care, from birth until the age of 6 years. Four different weight trajectories for both sexes combined were identified through normal mixture modeling for model-based clustering and labelled "normal weight gain", "weight gain during infancy", "weight gain during childhood" and "persistent weight gain", as previously described [26] (Supplemental figure 1).

Whole body DXA scans were performed in a Hologic Discovery QDR® 4500W device (Hologic Inc., Bedford, Massachusetts), according to standard manufacturer protocols in light clothing and without metal accessories. Total body less head (subtotal) and lumbar spine (LS) BMC was obtained and areal bone mineral density (aBMD) was expressed as BMC (in g) per projected bone area (in cm²) [27]. We performed daily standard quality assurance tests using the spine phantom. Scans were evaluated immediately after the scanning procedure and later validated by a second well-trained radiology technician.

Data analysis

For the description of reference intervals, according to the C28-A3 International Federation of Clinical Chemistry and Clinical Laboratory and Standards Institute guidelines (C28-A3 CLSI/IFCC guidelines), data were examined for outlier observations using frequency histograms and Dixon outlier range statistic as follows. Estimation of D/R ratio, where D is the absolute difference between the most extreme observation (large or small) and the next largest (or smallest) observation and R is the range of all observations (maximum-minimum) was estimated. If the ratio D/R exceeds 1/3, then the extreme observation is deleted, otherwise all datum is kept [28]. Using this procedure, no extreme values were detected as outliers for any of the bone markers distributions. Concentrations of bone metabolism markers are presented as medians, and the interval limits were determined by calculating the rank numbers for the 2.5th and 97.5th percentiles of the distribution [28]. The season during which blood samples were collected was combined into two categories: summer (April-September) and winter months (October-March).

Two-sample t test, Mann–Whitney test and chi-square test were used to compare boys and girls with regard to bone markers concentrations, age, anthropometrics (height, weight, BMI and trajectories of weight gain), 25(OH)D, PTHi, Ca and P_i.

Correlations were estimated using parametric statistics (Pearson correlation coefficients) to assess crude relationships among bone metabolism markers and between bone markers and age, height, weight and DXA-derived BMC and aBMD. To account for the effects of age, sex, body size (height and weight) and season on the relationships studied we computed Pearson partial correlations (r_{partial}). Correlations below 0.20 were interpreted as very weak, between 0.2 and 0.4 as weak and between 0.4 and 0.6 as moderate. Concentrations of bone markers were compared between BMI groups using Mann–Whitney test and between weight gain trajectories using ANOVA. Mean values of bone markers concentrations, adjusted for sex, age and season were estimated through ANCOVA and comparisons between BMI groups were performed using *ncom* command in Stata. Mean values of bone markers concentrations according to weight gain trajectories were additionally adjusted for current body size (height and weight at 7 years of age) and comparisons between trajectories were performed using Wald test (*testparm* command in Stata). Post-hoc pairwise comparison of adjusted means between trajectories were performed with the Tukey-Kramer correction for multiple comparison.

Sensitivity analyses

To assess the impact of vitamin D deficiency on the relationships between bone markers and anthropometrics and BMC/aBMD, we recalculated correlations after excluding children with serum 25(OH)D below 20 ng/ml [29] (n=153), and compared these estimates with those obtained for the whole sample. Additional sensitivity analyses to assess the impact of hypo- or hypercalcemia and hypo- or hyperphosphatemia were carried out by excluding children with serum Ca or P_i concentrations outside reference intervals established by the instrument manufacturer (Ca below 2.2 mmol/l or above 2.7 mmol/l, n=58; P_i below 1.292 mmol/l or above 2.261 mmol/l, n=3) [30].

All statistical analyses were conducted by using Stata version 11.2 for Windows (Stata Corp. LP, College Station, Texas, USA).

Results

Sample characteristics

In our sample of 7-year-old children, reference intervals expressed as 2.5th to 97.5th percentile were 159 to 439 U/l for serum tALP and 470 to 1690 ng/l for β -CTx. Since mean OC concentration was higher in girls than in boys (87.9 versus 82.1 μ g/l, respectively, p=0.003), reference intervals are presented separately by sex: 52.5 to 137.7 μ g/l in girls and 50.0 to 129.9 μ g/l in boys. Seasonal variation of bone metabolism markers was observed, with increased levels of tALP (p<0.001) and β -CTx (p=0.003) in children sampled in summer (n=314) than in those sampled in winter months (n=81) (Table 1).

There were no differences between girls and boys in age, weight and BMI distributions. Girls were slightly shorter than boys (122.7 versus 123.8 cm, p=0.022) had lower serum P_i concentration (1.63 versus 1.66 mmol/l, p=0.025), and were more likely to have a trajectory of weight gain during childhood. Serum vitamin D level was higher in samples of participants evaluated in summer than in winter months (p<0.001). Vitamin D insufficiency was present in 45.6% children while vitamin D deficiency was present in 38.7% children [29] (Table 1).

Table 1. Anthropometrics and serum concentrations of vitamin D, parathyroid hormone, calcium, phosphorus and bone metabolism markers, in girls and boys (n=395)

	TOTAL	Girls (n=197)	Boys (n=198)	Comparison between genders
	Mean $\pm$ SD or Median (P25, P75) or n (%)			p-value ^a
Age (months)	84.1 (83.6, 84.5)	83.9 (83.7, 84.5)	84.1 (83.6, 84.6)	0.674
Height (cm)	123.3 $\pm$ 5.1	122.7 $\pm$ 4.9	123.8 $\pm$ 5.2	0.022
Weight (kg)	25.2 (22.6, 28.1)	24.9 (22.6, 28.1)	25.2 (22.7, 28.2)	0.774
BMI categories (%) ^b				
Normal weight	262 (66.3)	127 (64.5)	135 (68.2)	0.719
Overweight	81 (20.5)	42 (21.3)	39 (19.7)	
Obese	52 (13.2)	28 (14.2)	24 (12.1)	
Growth trajectories (%)				
Normal weight gain	243 (67.5)	110 (61.1)	133 (73.9)	0.004
Weight gain during infancy	23 (6.4)	9 (5.0)	14 (7.8)	
Weight gain during childhood	54 (15.0)	38 (21.1)	16 (8.9)	
Persistent weight gain	40 (11.1)	23 (12.8)	17 (9.4)	
Missing data for growth trajectory assignment	35	17	18	
25(OH)D (ng/ml)				0.756
Total sample	21.8 (17.7, 26.3)	22.0 (17.7, 26.6)	21.7 (17.8, 26.0)	
Collected in summer ^c	23.0 (19.0, 28.0)	23.3 (19.1, 28.2)	22.6 (18.5, 27.8)	
Collected in winter ^d	17.2 (14.1, 21.1)	16.2 (12.9, 20.9)	18.0 (14.8, 21.8)	
Vitamin D insufficiency (%) ^e	180 (45.6)	83 (42.1)	97 (49.0)	

Vitamin D deficiency (%) ^e	153 (38.7)	81 (41.1)	72 (36.7)	0.583
PTHi (pmol/l)	3.24 (2.63, 3.83)	3.28 (2.65, 3.88)	3.20 (2.60, 3.74)	0.608
Ca (mmol/l)	2.5 (2.4, 2.6)	2.5 (2.4, 2.6)	2.5 (2.4, 2.6)	0.308
P_i (mmol/l)	1.64 ± 0.16	1.63 ± 0.16	1.66 ± 0.16	0.025
P50th (P2.5th - P97.5th)				
tALP (U/l) ^f				
Total sample	260 (159, 439)	258 (153, 439)	262 (167, 445)	0.820
Collected in summer ^g	269 (175, 445)	272 (167, 459)	265 (175, 445)	
Collected in winter ^d	228 (139, 396)	220 (134, 396)	239 (139, 584)	
OC (µg/l)				
Total sample	85.2 (50.3, 134.8)	87.9 (52.5, 137.7)	82.1 (50.0, 129.9)	0.003
Collected in summer ^c	85.6 (51.6, 136.5)	88.3 (52.5, 140.6)	82.2 (51.1, 130.2)	
Collected in winter ^d	83.6 (50.0, 124.3)	85.2 (59.6, 127.9)	81.6 (48.6, 114.5)	
β-CTx (ng/l)				
Total sample	1030 (470, 1690)	1040 (450, 1690)	1005 (510, 1690)	0.258
Collected in summer ^c	1040 (500, 1690)	1075 (450, 1700)	1025 (530, 1680)	
Collected in winter ^d	940 (470, 1380)	930 (500, 1320)	940 (440, 1700)	

^a Two-sample t-test, Mann–Whitney or chi-square tests as appropriate

^b According to World Health Organization reference data for BMI z-score: normal weight (≤ 1 SD), overweight (>1 SD and ≤ 2 SD) and obesity (>2 SD) (de Onis, *et al.*, Bull World Health Organ, 2007)

^c n=314 (154 Girls + 160 Boys)

^d n=81 (43 Girls + 38 Boys)

^e According to the Endocrine Society (United States), vitamin D insufficiency is defined as serum 25(OH)D of 21–29 ng/ml and vitamin D deficiency as serum 25(OH)D below 20 ng/ml (Holick, *et al.*, J Clin Endocrinol Metab, 2011)

^f n=394 (197 Girls + 197 Boys)

^g n=313 (154 Girls + 159 Boys)

Abbreviations: BMI, body mass index; 25(OH)D, 25-hydroxyvitamin D; PTHi, intact parathyroid hormone; Ca, calcium; P_i, inorganic phosphorus; tALP, total alkaline phosphatase; OC, osteocalcin; β-CTx, β-crosslaps

Relationship between bone remodeling markers

In both sexes, positive moderate correlations between OC and β -CTx were found, even after controlling for age, body size and season effects (girls: $r_{\text{partial}}=0.40$, 95% CI: 0.28, 0.51 and boys: $r_{\text{partial}}=0.46$, 95% CI: 0.34, 0.56). No relevant correlations were observed between tALP and OC (girls: $r_{\text{partial}}=0.02$, 95% CI: -0.12, 0.16 and boys: $r_{\text{partial}}=0.07$, 95% CI: -0.07, 0.21) or between tALP and β -CTx (girls: $r_{\text{partial}}=0.13$, 95% CI: -0.01, 0.27 and boys: $r_{\text{partial}}=0.00$, 95% CI: -0.14, 0.14) (Table 2).

Table 2. Pearson correlation and partial correlation coefficients among bone metabolism markers, in girls and boys (n=395)

		Girls (n=197)					
		tALP		OC		β -CTx	
		Coefficient	95% CI	Coefficient	95% CI	Coefficient	95% CI
Boys (n=198)	tALP			r=0.06	-0.08, 0.20	r=0.18	0.04, 0.31
				$r_{\text{partial}}=0.02$	-0.12, 0.16	$r_{\text{partial}}=0.13$	-0.01, 0.27
	OC	r=0.11	-0.03, 0.25			r=0.40	0.28, 0.51
		$r_{\text{partial}}=0.07$	-0.07, 0.21			$r_{\text{partial}}=0.40$	0.28, 0.51
	β -CTx	r=0.06	-0.08, 0.20	r=0.47	0.36, 0.58		
		$r_{\text{partial}}=0.00$	-0.14, 0.14	$r_{\text{partial}}=0.46$	0.34, 0.56		

Abbreviations: tALP, total alkaline phosphatase; OC, osteocalcin; β -CTx, β -crosslaps; r, Pearson correlation coefficient; r_{partial} , Pearson partial correlation coefficient (age, body size and season controlled); 95% CI, 95% confidence interval.

Bone metabolism markers and age and anthropometrics

Correlations between age and tALP and β -CTx were very weak and attenuated after accounting for sex and season effects ($r_{\text{partial}}=0.14$, 95% CI: 0.04, 0.23 and $r_{\text{partial}}=0.08$, 95% CI: -0.02, 0.17, respectively) (Fig 1a and Fig 1c).

As regards anthropometrics, tALP was slightly correlated with height ($r=0.30$, 95% CI: 0.20, 0.38) (Fig 1d) and weight ($r=0.26$, 95% CI: 0.16, 0.35) (Fig 1g) while correlations between height or weight and OC or β -CTx were irrelevant (Fig 1e, Fig 1f, Fig 1h and Fig 1i). After accounting for the effect of sex, age and season, the positive relationship between height and tALP was attenuated ($r_{\text{partial}}=0.26$, 95% CI: 0.17, 0.35) (Fig 1d). Comparing BMI groups, we observed that overweight children had higher tALP concentration than normal weight children [mean (95% CI) adjusted for sex, age and season: 287 (275, 300) versus 263 (254, 272) U/l, $p=0.002$] (Fig 1j). Also for tALP, we

observed that, in comparison to children in a “normal weight gain” trajectory [mean (95% CI) adjusted for sex, age and season: 268 (259, 278) U/l], those in the trajectories of “weight gain during infancy” presented slightly higher serum concentrations [308 (278, 339) U/l, $p=0.018$] (Fig 1m). For OC and β -CTx, we observed no differences in serum concentrations by BMI groups or by trajectories of weight gain (Fig 1k, Fig 1l, Fig 1n and Fig 1o).

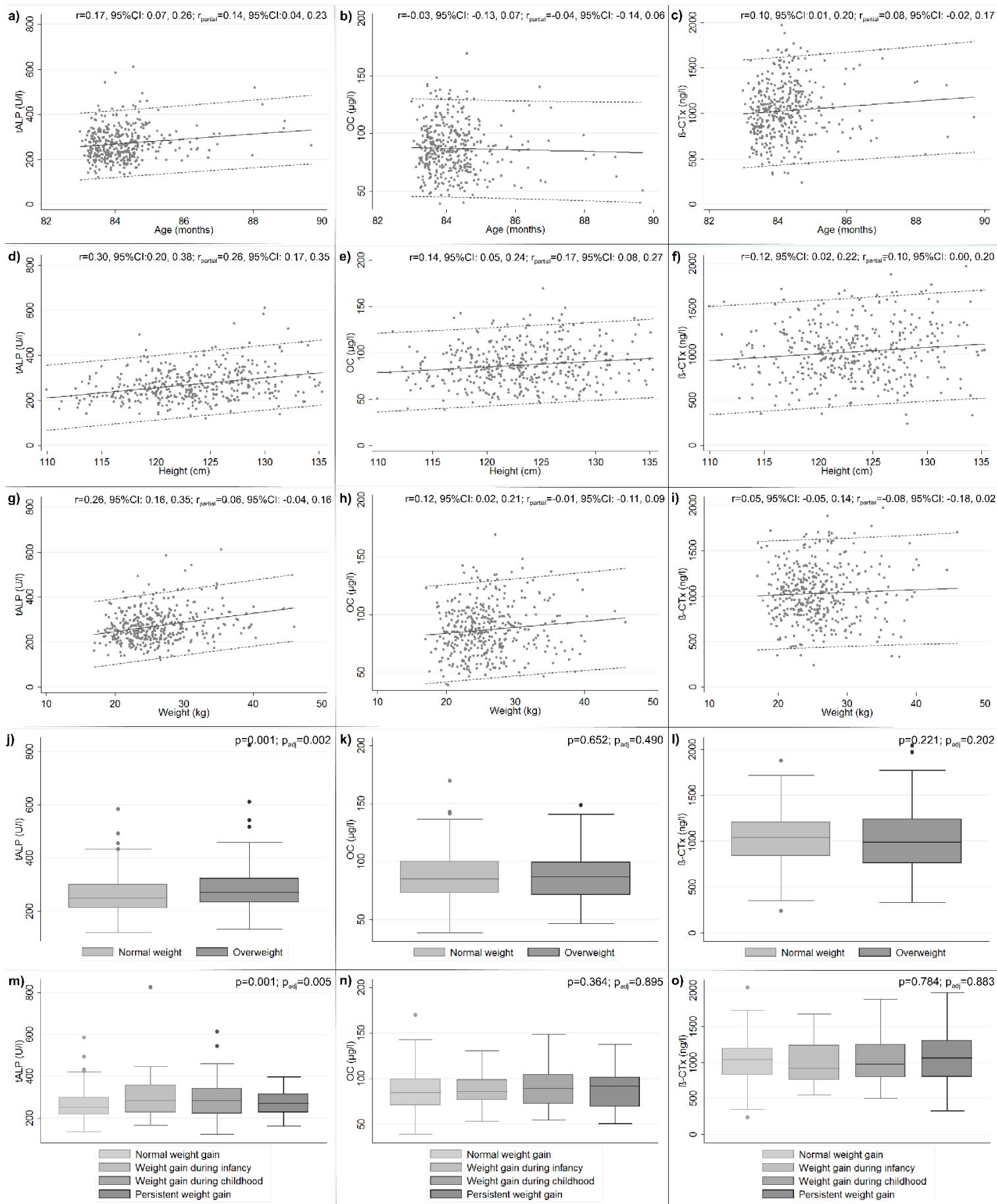


Fig 1. Serum concentrations of bone metabolism markers in relation to age and anthropometrics (n=395). (a) tALP in relation to age. (b) OC in relation to age. (c) β -CTx in relation to age. (d) tALP in relation to height. (e) OC in relation to height. (f) β -CTx in relation to height. (g) tALP in relation to weight. (h) OC in relation to weight. (i) β -CTx in relation to weight. (j) tALP by BMI groups. (k) OC by BMI groups. (l) β -CTx by BMI groups. (m) tALP by trajectories of weight gain. (n) OC by trajectories of weight gain. (o) β -CTx by trajectories of weight gain.

Panels a) to i): the solid line represents the mean and the dotted lines $\pm$ 2SD; r , Pearson correlation coefficient, r_{partial} , Pearson partial correlation coefficient (correlations between bone metabolism markers and age were controlled for sex and season; between bone metabolism markers and height were controlled for sex, age and season; between bone metabolism markers and weight were controlled for sex, age, height and season). For better data visualization, the participant with 95months of age is not represented in Panels a) to c).

Panels j) to l): p , p -values from the Mann–Whitney test for comparisons between BMI groups; p_{adjusted} , p -values from the *nlcom* postestimation command for comparisons between BMI groups (means of bone metabolism markers were adjusted for sex, age and season).

Panel m) to o): p , p -values from ANOVA for comparisons between trajectories of weight gain; p_{adjusted} , p -values from the *testparm* Stata command for comparisons between trajectories of weight gain (means of bone metabolism markers were adjusted for sex, age, current body size and season).

Abbreviations: tALP, total alkaline phosphatase; OC, osteocalcin; β -CTx, β -crosslaps; BMI, body mass index, 95% CI, 95% confidence interval.

Bone metabolism markers and DXA-derived bone measures

Overall, Table 3 shows that correlations between bone metabolism markers and DXA-derived BMC and aBMD were positive but modest in magnitude. Crude correlations with BMC and aBMD were slightly stronger for tALP than for OC or β -CTx. Serum OC was slightly stronger correlated with BMC/aBMD in girls than in boys. However, after computing partial correlations accounting for age, body size and season effects, tALP was no longer correlated with bone mass, neither in girls nor in boys. In girls only, weak positive correlations between OC and subtotal BMC ($r_{\text{partial}}=0.22$, 95% CI: 0.08, 0.35), subtotal aBMD ($r_{\text{partial}}=0.20$, 95% CI: 0.06, 0.33) and lumbar spine aBMD ($r_{\text{partial}}=0.23$, 95% CI: 0.09, 0.36) were observed. β -CTx was not correlated with any of the DXA-derived bone measures.

Table 3. Pearson correlation and partial correlation coefficients between bone metabolism markers and bone mineral content and density, in girls and boys (n=395)

		Subtotal BMC		Lumbar spine BMC		Subtotal aBMD		Lumbar spine aBMD	
		Coefficient	95% CI	Coefficient	95% CI	Coefficient	95% CI	Coefficient	95% CI
tALP	Girls	$r=0.25$	0.12, 0.38	$r=0.18$	0.05, 0.32	$r=0.24$	0.11, 0.37	$r=0.19$	0.06, 0.33
		$r_{\text{partial}}=0.02$	-0.12, 0.16	$r_{\text{partial}}=0.08$	-0.06, 0.22	$r_{\text{partial}}=0.02$	-0.12, 0.16	$r_{\text{partial}}=0.09$	-0.05, 0.23
	Boys	$r=0.24$	0.11, 0.37	$r=0.22$	0.08, 0.35	$r=0.22$	0.08, 0.35	$r=0.20$	0.06, 0.33
		$r_{\text{partial}}=0.11$	-0.03, 0.25	$r_{\text{partial}}=0.13$	-0.01, 0.27	$r_{\text{partial}}=0.10$	-0.04, 0.23	$r_{\text{partial}}=0.13$	-0.01, 0.26
OC	Girls	$r=0.23$	0.09, 0.36	$r=0.14$	0.00, 0.28	$r=0.20$	0.06, 0.33	$r=0.26$	0.12, 0.38
		$r_{\text{partial}}=0.22$	0.08, 0.35	$r_{\text{partial}}=0.10$	-0.04, 0.23	$r_{\text{partial}}=0.20$	0.06, 0.33	$r_{\text{partial}}=0.23$	0.09, 0.36
	Boys	$r=0.19$	0.05, 0.32	$r=0.06$	-0.08, 0.20	$r=0.14$	0.00, 0.28	$r=0.13$	-0.01, 0.27
		$r_{\text{partial}}=0.08$	-0.06, 0.21	$r_{\text{partial}}=-0.07$	-0.21, 0.07	$r_{\text{partial}}=0.04$	-0.10, 0.18	$r_{\text{partial}}=0.06$	-0.08, 0.19
β-CTx	Girls	$r=0.07$	-0.07, 0.21	$r=0.08$	-0.06, 0.22	$r=0.01$	-0.13, 0.15	$r=0.04$	-0.10, 0.18
		$r_{\text{partial}}=0.11$	-0.03, 0.25	$r_{\text{partial}}=0.04$	-0.10, 0.18	$r_{\text{partial}}=0.03$	-0.11, 0.17	$r_{\text{partial}}=0.05$	-0.09, 0.19
	Boys	$r=0.10$	-0.04, 0.24	$r=0.07$	-0.07, 0.21	$r=0.06$	-0.08, 0.20	$r=0.09$	-0.05, 0.22
		$r_{\text{partial}}=0.04$	-0.10, 0.18	$r_{\text{partial}}=-0.02$	-0.16, 0.12	$r_{\text{partial}}=-0.00$	-0.14, 0.14	$r_{\text{partial}}=0.06$	-0.08, 0.19

Abbreviations: tALP, total alkaline phosphatase; OC, osteocalcin; β -CTx, β -crosslaps; BMC, bone mineral content; aBMD, areal bone mineral density; r , Pearson correlation coefficient; r_{partial} , Pearson partial correlation coefficient (age, body size and season controlled); 95% CI, 95% confidence interval.

Sensitivity analyses

After excluding children with vitamin D deficiency (n=153), with hypo- and hypercalcemia (n=58) or hypo- and hyperphosphatemia (n=3), one group at a time, correlation coefficients between bone metabolism markers and anthropometrics and DXA-derived bone measures remained similar to those obtained for the whole sample of children before exclusion of these subjects.

Discussion

In this study, we described reference intervals for tALP, OC and β -CTx in a population-based sample of 7-year-old children. We found a moderate correlation between serum concentrations of bone-specific metabolism markers OC and β -CTx, likely representing the dynamic nature of bone turnover. The non-specific bone marker tALP was slightly more correlated than OC or β -CTx with bone mass and anthropometric variables, probably reflecting the overall trajectory of anthropometric growth up to the time of measurement.

There has been growing interest in the quantification of bone metabolism markers in the clinical setting as they may provide a dynamic, short-term measure of skeletal status, which is not captured by bone physical properties alone [2]. So far, no single individual parameter has fulfilled all the criteria for an ideal marker of bone formation or resorption [6]. The clinical utility of bone markers is challenged by high intraindividual variation, lack of specificity for bone tissue, release during different anabolic and catabolic processes and influence of non-skeletal processes on circulating levels [31]. Also, no single marker is sufficiently precise to be used for prognostic purposes, as concentration changes of candidate molecules are neither site- nor disease-specific [6]. An additional challenge is posed in the interpretation of bone metabolism markers as a prognostic tool, in populations of children and adolescents, because it is impossible to distinguish treatment-induced changes from the physiological age-related decline in these bone markers [31]. Furthermore, some bone metabolism markers, such as OC, may reflect, at the same time, both bone formation and resorption processes [31]. Because there is no specific bone marker to assess bone modeling, remodeling or epiphyseal growth, concentrations of markers in children represent the combined effects of these different biological processes [6]. This means that equal serum levels of bone metabolism markers can be found both in children with high bone remodeling and low rate of growth as well as in normally growing children [32].

In addition to limitations in inferring the biological significance of bone metabolism markers, the definition of pediatric reference intervals is limited by their substantial pre-analytical variability and by heterogeneity in analytical methods [8]. Pre-analytical sources of variability include uncontrollable factors (such as sex, age, pubertal development, growth velocity, ethnicity, physical activity, nutritional status, or pathological conditions (e.g. diabetes, liver diseases, growth hormone deficiency, vitamin D deficiency, or recent fracture) and drugs), and controllable factors such as circadian rhythm, seasonal variation or fasting status [8]. The main sources of undesired pre analytical variability of tALP are related to age, pubertal status and sex, as well as lack of bone specificity [32]. Regarding OC, pre-analytical variability is mostly related to age and circadian rhythm [8]. β -CTx also exhibits significant circadian rhythm and is influenced by food intake, as well as age [8]. Analytical variability further contributes to variation of reference intervals, due to lack of standardization and harmonization of quantification methods and to inter-laboratory variation, even when the same method is used [33]. Attempts to establish reference intervals have also been limited by the small sample sizes of most previous investigations, which have also been frequently conducted in hospital settings [33]. Indeed, reference values obtained from facility-based samples may not be applicable to the general pediatric population as intervals in the former are generally wider [34].

To account for those limitations, our approach in the present study was to use data from a comparatively large population-based sample of children of the same age, whose samples were drawn in the morning, after an overnight fast. Nevertheless, our reported reference values are directly applicable only to children of the same age whose samples were tested using laboratory methods similar to ours [28], as supported by our comparison of the present results with previous descriptions (Supplemental tables 1 to 3). Regarding 25(OH)D, PTHi, Ca and P_i we found that concentrations in our sample were within published reference intervals although no previous studies have reported sex differences in serum concentration of P_i [35, 36]. However, our findings on the relationships between bone metabolism markers and anthropometrics or DXA-derived bone measures are more dependent on the relative positions of children in the distribution of each marker and less on absolute value of marker concentrations, which makes for safer generalization of the magnitude of associations to other contexts.

We found a positive correlation between OC and β -CTx, in agreement with findings in other samples of prepubertal children [17] but in contrast with one study including 5 to 10 year-old children [14]. Positive relationships between bone-specific formation and

resorption markers are expected, and reflect the nature of bone turnover as a tightly regulated dynamic mechanism of formation and resorption [6]. Our absence of correlation between tALP and OC corroborates previous findings [37] but contradicts more recent results that showed direct correlations among formation markers [13, 38]. The recent hypothesis that OC is a marker of bone turnover as a whole, reflecting not only bone formation but also resorption, may explain its less consistent associations with other formation markers [39]. Our results also differed from previous findings that disclosed a positive relationship between ALP and β -CTX. However, other studies have assessed bone-specific ALP rather than total ALP [38]. In addition, since previous studies have looked at wider age ranges, results are not straightforwardly comparable to ours in children of the same age.

Overall, weak correlations among bone markers are probably due to the coexistence of different biological processes at different anatomical regions and bone surfaces during skeletal growth. In addition, markers are released during different stages of bone formation and resorption, and have different elimination pathways and serum half-lives, which may affect their relations at different time points during growth [6].

Our results were indicative of a weak positive effect of age on tALP concentrations, which corroborates previous findings of an age-related increase in tALP, expected to peak at approximately 10-12 years of age in girls and 13-15 years in boys, and detectable around 7 years of age [35, 36, 40-43]. At this age, tALP was slightly correlated with height. Also, higher concentrations were observed in overweight when compared to healthy weight participants, and in children who gained weight above-average during infancy, in comparison to those in an average weight gain trajectory. These modest positive relationships between tALP and anthropometric growth, which were not apparent for the bone-specific markers OC and β -CTX, suggest that tALP is a more specific marker of the child's overall growth trajectory up to the time of measurement. Positive correlations between bone-specific ALP and anthropometric measures have been reported elsewhere [38] while others shown no effect of BMI on tALP values [44]. Our results did not support previously described positive correlations between OC and height and weight [10, 14], between CTX and weight [14] and height [13, 38] or the finding of decreased OC values in obese when compared to healthy weight children [11, 12]. However, in accordance to our findings, a number of other studies have contributed with evidence of a lack of association between height, weight or BMI and OC [9, 10, 13, 14, 39, 44] and height or BMI and β -CTX [44]. Increased formation markers in taller and heavier children are likely reflecting greater growth velocity, inducing increased bone formation in

response to greater mechanical strain [13, 32]. Accordingly, CTX may also correlate with height since increased periosteal modeling also implies removal of bone at the endocortical surface [45]. To the best of our knowledge, our study is the first to estimate relationships between bone markers and growth trajectories from birth. Given the lack of relationships between OC or β -CTX and trajectories of weight gain we believe that, contrarily to dimensions of skeletal growth, such as BMC, that are clearly shaped by the overall weight trajectory, markers of bone metabolism are not related to overall weight trajectories [46]. The lack of associations between OC or β -CTX and the remaining anthropometric growth indices supports this assertion.

Bone metabolism markers were also only weakly correlated with DXA-derived BMC and aBMD. Additional adjustments for age, body size and season attenuated those relationships only for OC and only in girls. This suggests that measurements of bone metabolism markers at a single point in time do not reflect bone mineralization status in the general prepubertal population and are unlikely to be helpful for monitoring the status of bone mineral accrual in non-clinical settings. These findings are consistent with the premise that bone markers reflect instantaneous metabolic activity and do not directly translate physical dimensions that result from the cumulative process of bone gain throughout several years [6]. In prepubertal children, some previous studies have also found no relationship between tALP, OC or β -CTX and whole body (WB) and LS BMC or BMD [14, 15, 35]. Other studies disclosed, however, consistent positive relationships between tALP and OC and WB and LS BMC, in boys [16] and between OC and CTX and WB and LS BMC/BMD, in both sexes [15, 17]. In older pubertal children, inverse correlations between serum OC or CTX and BMD were disclosed [9, 15], which is expected because, during puberty, bone turnover decreases with advancing sexual maturation while, at the same time, bone mass accrual continues up to peak bone mass [7]. Some authors have also described site-specific associations between bone metabolism markers and bone mass during puberty, disclosing stronger relationships in anatomical sites with more trabecular bone (e.g. LS BMC) than with cortical bone (FN BMC), possibly due to higher metabolic activity of trabecular when compared to cortical bone [47]. In our study, however, bone markers were not correlated with either cortical or trabecular bone when we computed correlations between bone markers and BMC and aBMD at the LS and lower limbs (S1 File, S4 Table).

This study extends previous evidence by assessing the usefulness of bone markers to describe growth in a population-based sample of children with longitudinal anthropometric data. In particular, we were able to investigate for the first time the

relationships of bone markers with weight gain trajectories since birth. We also quantified bone metabolism markers at the same chronological age for all participants. Furthermore, blood samples were all collected in the morning after overnight fasting to avoid the variability associated to the circadian rhythm. In addition, reference intervals were described according the statistical approach recommended in the C28-A3 CLSI/IFCC guidelines [28]. However, we should acknowledge a limitation in our use of tALP instead of bone-specific ALP. Serum variations of tALP are not as accurate as bone-specific ALP to detect subtle changes in bone formation [6]. Nevertheless, when liver ALP is stable and remains within normal values, tALP represents a valid marker of bone turnover [31].

In the future, it will be interesting to investigate time changes in bone markers and whether those are associated with changes in bone mass and density across the lifespan. In particular, it might be important to explore how puberty relates to bone formation and resorption. Although we were not able to assess sexual development in this evaluation, we expect that the vast majority of children from the general population were prepubertal at 7 years of age, as observed in previous population-based samples of the same age [48, 49].

In conclusion, we found weak or negligible associations of bone metabolism markers with different indices of growth, suggesting limited ability of those markers to predict bone mass and overall growth in 7-year-old children from the general population.

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Supplemental table 1. Reference intervals for serum total alkaline phosphatase concentrations (U/l)

First author, year	Age (years)	N	Analytic platform	P50 (P2.5, P97.5)
Both sexes				
<i>Generation XXI, 2019</i>	7	393	Beckman Coulter Olympus AU5400 analyzer	260 (159, 439)
<i>Wanjian, 2017 [1]</i>	6-12	175	Beckman Coulter AU5800 analyzer	(48.8, 445.9)
<i>Abou El Hassan, 2015 [2]</i>	1-10	391	Beckman Coulter AU analyzer	(160, 381)
<i>Buchanan, 2015 [3]</i>	5-13	121	Cobas Integra 400 plus analyzer	278 (IC95%: 174, 460)
<i>Ridefelt, 2014 [4]</i>	2-8	234	Abbott Architect ci8200 analyzer	(111, 277)
<i>Hilsted, 2013 [5]</i>	7-8	228	Roche Modular Analytics P/ISE-System analyzer	(154, 358)
Girls				
<i>Generation XXI, 2019</i>	7	197	Beckman Coulter Olympus AU5400 analyzer	258 (153, 439)
<i>Zierk, 2017 [6]</i>	7	3785	Roche Cobas analyzer	217 (127, 345)
<i>Cho, 2014 [7]</i>	6-12	988	Hitachi 7600 analyzer	(123, 330)
<i>Hilsted, 2013 [5]</i>	7-8	135	Roche Modular Analytics P/ISE-System analyzer	(157, 365)
<i>Huang, 2011 [8]</i>	6-11	36	Roche P-modular analyzer	(157, 359)
Boys				
<i>Generation XXI, 2019</i>	7	196	Beckman Coulter Olympus AU5400 analyzer	262 (167, 445)
<i>Zierk, 2017 [6]</i>	7	4237	Roche Cobas analyzer	208 (125, 323)
<i>Cho, 2014 [7]</i>	6-12	1094	Hitachi 7600 analyzer	(118, 341)
<i>Hilsted, 2013 [5]</i>	7-8	93	Roche Modular Analytics P/ISE-System analyzer	(149, 345)
<i>Huang, 2011 [8]</i>	6-15	75	Roche P-modular analyzer	(136, 414)

Supplemental table 2. Reference intervals for serum osteocalcin concentrations (µg/l)

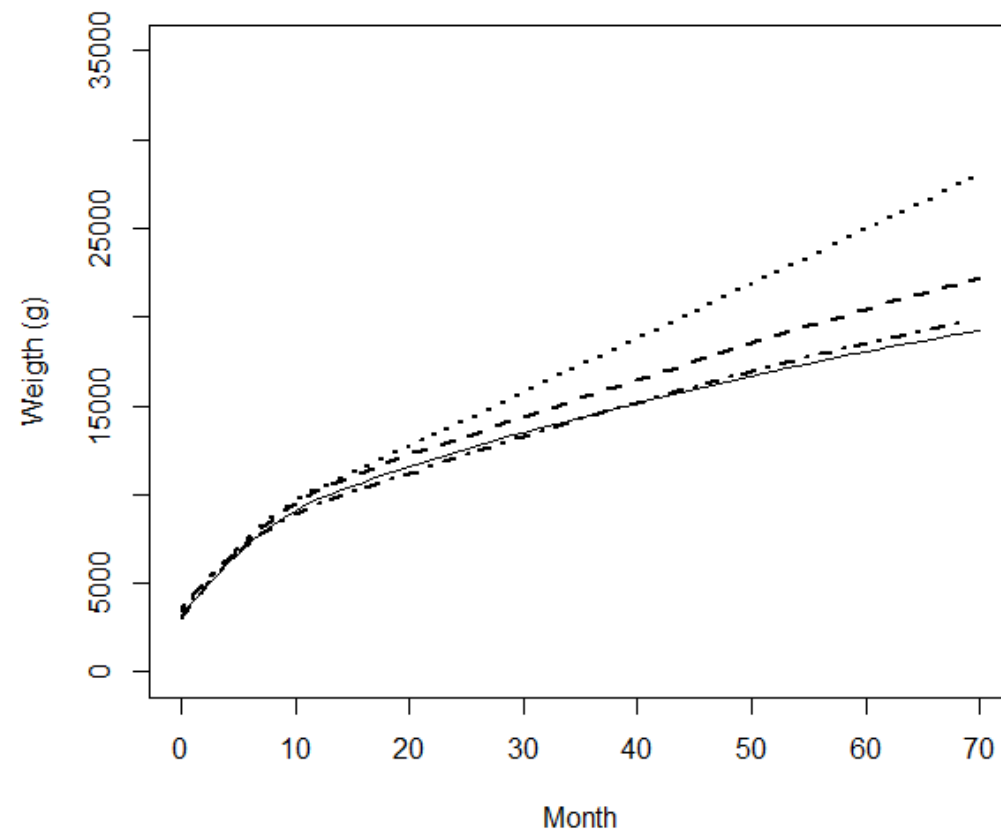
First author, year	Age (years)	N	Assay/Analytic platform	P50 (P2.5, P97.5)
Both sexes				
<i>Generation XXI, 2019</i>	7	395	Roche Cobas e411 analyzer	85.2 (50.3, 134.8)
<i>Bayer, 2014 [9]</i>	7.1 - 8	15	Roche Elecsys analyzer	(12.5, 232.5)
<i>Manjon, 2004 [10]</i>	4-10	25	Metra Biosystems NovoCalcin assay	Mean (IC95%): 15.128 (12.30, 17.95)
Girls				
<i>Generation XXI, 2019</i>	7	197	Roche Cobas e411 analyzer	87.9 (52.5, 137.7)
<i>Huang, 2011 [8]</i>	6-10	33	Roche P-modular analyzer	(61.4, 136.2)
<i>Rauchenzauner, 2007 [11]</i>	7	32	Active Human Osteocalcin IRMA	19.96 (P3, P97: 7.90, 50.44)
<i>Vietri, 2006 [12]</i>	6.1-8	207	DiaSorin LIASON analyzer	Mean: 69.06 (31.46, 92.10)
<i>Seydewitz, 2001 [13]</i>	6-8	25	Immulinite immunoanalyzer	31.3 (P5, P95: 18.6, 62.0)
<i>Cioffi, 1997 [14]</i>	7	42	CIS Bio International IRMA	72.6 (P25, P75: 53, 97)
Boys				
<i>Generation XXI, 2019</i>	7	198	Roche Cobas e411 analyzer	82.1 (50, 129.9)
<i>Huang, 2011 [8]</i>	6-9	30	Roche P-modular	(56.5, 152.1)
<i>Rauchenzauner, 2007 [11]</i>	7	32	Active Human Osteocalcin IRMA	19.54 (P3, P97: 8.12, 47.00)
<i>Vietri, 2006 [12]</i>	6.1-8	209	DiaSorin LIASON analyzer	Mean: 65.6 (27.48, 98.36)
<i>Seydewitz, 2001 [13]</i>	6-8	34	Immulinite immunoanalyzer	31.6 (P5, P95: 10.6, 50.5)
<i>Cioffi, 1997 [14]</i>	7	51	CIS Bio International IRMA	66.8 (P25, P75: 49, 104)

Supplemental table 3. Reference intervals for serum β -crosslaps concentrations (ng/l)

First author, year	Age (years)	N	Assay/Analytic platform	P50 (P2.5, P97.5)
Both sexes				
<i>Generation XXI, 2019</i>	7	395	Roche Cobas e411 analyzer	1030 (470, 1690)
<i>Crofton, 2002 [15]</i>	1-9	124	Osteometer Biotech ELISA	352 (95% CI: 146, 818)
Girls				
<i>Generation XXI, 2019</i>	7	197	Roche Cobas e411 analyzer	1040 (450, 1690)
<i>De Melo, 2018 [16]</i>	6-9	23	Roche Cobas e170 analyzer	(565, 1570)
<i>Gennai, 2016 [17]</i>	7	NA	Technogenetics-Bouty DSX system	2000 (P10, P90: 500, 3000) ^a
<i>Herrmann, 2014 [18]</i>	7.5	186	Roche ECLIA Modular E17	1260 (P3, P97: 750, 1770) ^b
<i>Alberti, 2011 [19]</i>	7-8	NA	Immunodiagnostic systems ELISA	2078 (1413, 3410)
<i>Huang, 2011 [8]</i>	6-10	33	Roche P-modular	(820, 2060)
<i>Rauchenzauner, 2007 [11]</i>	7	32	Osteometer Biotech One Step ELISA	1836 (P3, P97: 897, 3109)
Boys				
<i>Generation XXI, 2019</i>	7	198	Roche Cobas e411 analyzer	1005 (510, 1690)
<i>De Melo, 2018 [16]</i>	6-9	28	Roche Cobas e170 analyzer	(509, 1697)
<i>Gennai, 2016 [17]</i>	7	NA	Technogenetics-Bouty DSX system	1500 (P10, P90: 300, 3300) ^a
<i>Herrmann, 2014 [18]</i>	7.0-7.9	198	Roche ECLIA Modular E17	1230 (P3, P97: 750, 1710) ^b
<i>Alberti, 2011 [19]</i>	7-8	NA	Immunodiagnostic systems ELISA	2151 (1285, 2902)
<i>Huang, 2011 [8]</i>	6-9	29	Roche P-modular	(1050, 2380)
<i>Rauchenzauner, 2007 [11]</i>	7	32	Osteometer Biotech One Step ELISA	1637 (P3, P97: 787, 2794)

^a Abstracted from visual inspection of the reference curves

^b Assuming a mean height of 126 cm in girls and 127 cm in boys



Supplemental figure 1. Weight trajectories in the Generation XXI cohort

Solid line, “normal weight gain” trajectory; dashed-dotted line, “weight gain during infancy” trajectory; dashed line, “weight gain during childhood” trajectory; dotted line, “persistent weight gain” trajectory.

Supplemental table 4. Pearson correlation and partial correlation coefficients between bone metabolism markers and bone mineral content and density in lower limbs, in girls and boys (n=395)

		Lower limbs BMC		Lower limbs aBMD	
		Coefficient	95% CI	Coefficient	95% CI
tALP	Girls	r=0.26	0.12, 0.38	r=0.25	0.12, 0.38
		$r_{\text{partial}}=0.04$	-0.11, 0.17	$r_{\text{partial}}=0.04$	-0.10, 0.18
	Boys	r= 0.26	0.13, 0.39	r=0.22	0.08, 0.35
		$r_{\text{partial}}=0.15$	0.01,0.28	$r_{\text{partial}}=0.09$	-0.05, 0.23
OC	Girls	r=0.25	0.11, 0.38	r=0.18	0.04, 0.31
		$r_{\text{partial}}=0.25$	0.12, 0.38	$r_{\text{partial}}=0.16$	0.02, 0.30
	Boys	r=0.18	0.04, 0.31	r=0.14	0.00, 0.28
		$r_{\text{partial}}=0.06$	-0.08, 0.20	$r_{\text{partial}}=0.05$	-0.08, 0.19
β-CTx	Girls	r=0.08	-0.06, 0.22	r=0.01	-0.13, 0.15
		$r_{\text{partial}}=0.11$	-0.03, 0.25	$r_{\text{partial}}=0.04$	-0.10, 0.18
	Boys	r=0.09	-0.05, 0.22	r=0.06	-0.08, 0.20
		$r_{\text{partial}}=0.00$	-0.14, 0.14	$r_{\text{partial}}=0.00$	-0.14, 0.14

Abbreviations: tALP, total alkaline phosphatase; OC, osteocalcin; β-CTx, β-crosslaps; BMC, bone mineral content; aBMD, areal bone mineral density; r, Pearson correlation coefficient, r_{partial} , Pearson partial correlation coefficient (age, body size and season controlled), 95% CI, 95% confidence interval.

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4.6. When to Stop Looking at a Cause: an Application to Adiposity and Bone Physical Properties in Children

Raquel Lucas, Teresa Monjardino

Abstract

Background: We tested the potential for adiposity to specifically influence bone mass in children, using the association between socioeconomic position (SEP) and fat mass as a benchmark.

Methods: We used data from 2408 7-year-old children born in 2005/6, recruited as part of the Generation XXI cohort (Porto, Portugal). Weight and height were measured using standard methods and body composition (fat mass, lean mass and bone mineral content) was estimated using whole-body dual-energy X-ray absorptiometry. Socioeconomic position was characterized using 10 context-appropriate indicators: caregivers' educational level and occupation, household income, child's type of school, caregiver smoking, maternal smoking during pregnancy, hiring a housekeeper and taking holidays away. Associations were estimated using linear regression coefficients and 95% confidence intervals. Sensitivity analysis was conducted by excluding children more susceptible to nonparticipation or attrition.

Results: By design, all SEP indicators were associated with fat mass. Bone mineral content was associated with the educational level of the main (less than higher vs. higher education, standardized coefficient and 95% CI: 0.15; 0.07, 0.24) and secondary (0.13; 0.03, 0.22) caregivers and with the occupations of the main (blue and lower white vs. upper white collar: 0.12; 0.03, 0.20) and secondary (0.10; 0.01, 0.18) caregivers. Associations were negligible with the six remaining SEP indicators. A similar pattern was found for lean mass and height. Sensitivity analysis did not change our findings.

Conclusions: Using social causes as benchmark, fat mass likely has a small specific effect on bone mineral content beyond its contribution to body size in childhood.

Introduction

Fragility fractures are, and will likely remain, frequent causes of death and impairment in older adults [1,2]. Bone physical properties have emerged as a useful proxy for fracture risk since they convey the resistance of bone to external trauma in addition to other less well defined components of frailty [3]. An additional practical advantage of studying physical properties of bone is the possibility to estimate these using noninvasive methods long before fracture events are expected but when prevention strategies might be the most effective [4,5].

Of all the evidence that has been produced on the causes of bone fragility, arguably the most consistently reported besides age is body size [6]. At any given age, it is a generalized observation that larger individuals have higher bone mineral density and content [7,8]. This provides empirical support for theoretical models of bone mineralization that place mechanical demands as the main osteogenic stimulus, particularly during growth [9]. Changes to body size are seen as challenges to mechanical stability that induce changes in bone mass and architecture to better adapt the skeleton to the load it bears [6]. In view of the overwhelming evidence against excess adiposity, this makes for a very particular population health challenge since it implies that higher weight leads to improved bone properties [10]. As a consequence, several epidemiologic investigations, including our own previous work, have attempted to isolate endocrine effects of adiposity on bone properties using explicit or implicit structural modeling [11-13]. After statistically accounting for weight, most studies have concluded for a small but deleterious effect of adiposity on bone physical properties [14], typically discussed on the basis of physiological mechanisms linking fat and bone metabolism [15].

We believe that any estimation of an average direct effect of fat on bone that statistically discounts its weight component raises important validity concerns. Even if we use the unrealistic assumptions of no unmeasured confounding by common causes of adiposity and weight, and no collider bias due to conditioning on weight (Figure 1), the close biological dependence between weight and adiposity has clear potential for sparse data bias: it is very implausible, for instance, that we find empirical data from individuals with high fat mass but low body weight [16]. These common challenges illustrate the limitations of using epidemiology to disentangle physiological processes, which are not its object [17].

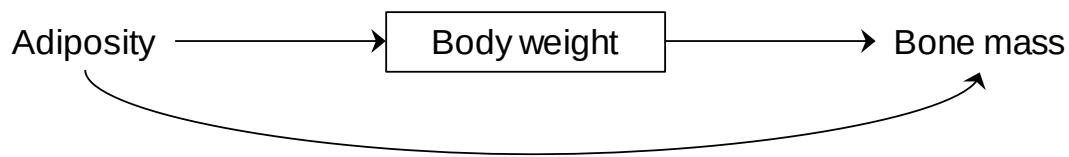


Figure 1. Diagram depicting hypothesized causal relations between adiposity, body weight and bone mass in the implausible scenario of 1) no unmeasured confounding by common causes of adiposity and body weight, and 2) no collider bias due to conditioning on weight as an intermediate step. In this unlikely scenario, body weight adjustment would suffice to estimate the specific (weight-independent) effect of adiposity on bone mass.

An alternative straightforward approach that seems more coherent with epidemiologic thinking is to use common causes as a benchmark. In most high-income countries, the distribution of adiposity is socially-patterned, whereas this effect is currently a lot less marked for other body size and composition traits, such as height and lean mass [18]. If adiposity is a specific determinant of bone mass beyond its contribution to body size, we expect that specific causes of adiposity (i.e. that are less relevant for other constitutional traits) are also indirect causes of bone mass, to a measurable extent. If this is the case, the associations between socioeconomic position (SEP) indicators and bone mass should mirror those between SEP indicators and fat mass. Evidence to the contrary may be interpreted as a lack of specificity of fat as a determinant of bone mass.

Therefore, in this study we aimed to indirectly test the potential for adiposity to specifically influence bone physical properties in children, by assessing whether well-documented socioeconomic causes of fat mass are associated with bone mineral content in a birth cohort of 7-year-old children.

Methods

Study population and sample

This study was conducted within Generation XXI, a birth cohort study with a geographically defined target population. All women who delivered live-born children with more than 23 weeks of gestation in one of the five public units providing level III obstetrical and neonatal care in the metropolitan area of Porto, Portugal, between April 2005 and August 2006, and whose residence was in the units' catchment area were eligible for recruitment. Of those invited, 91.4% accepted to participate. All 8647 children enrolled at birth were invited to attend follow-up assessments at ages four (2009-2011) and seven (2012-2014) years (86.3% and 79.7% participation, respectively). Children were typically invited for evaluations up to 8 weeks after their birthdays. Ethical approval was obtained from the Ethics Committee of the *Hospital de São João* and the University of Porto Medical School. The study protocol is registered with the Portuguese Data Protection Authority and conforms to the Helsinki Declaration and national legislation. At each evaluation, written informed consent was obtained from all legal guardians and oral assent was obtained from children. Detailed procedures have been published elsewhere [19,20]. Questionnaires in Portuguese are available online: <http://www.geracao21.com/pt/projeto#avaliacoes>.

This study was conducted in a consecutive subsample of participants who attended the face-to-face interviews at age seven between December 1, 2012 and August 31, 2013 and were invited to perform a whole-body dual-energy X-ray absorptiometry (DXA) scan. The selection of this subsample was due to funding schedule and equipment availability. Of the 3015 eligible participants, DXA scans were conducted successfully in 2408 children (257 explicitly refused to undergo the scan, 335 did not show up to any of three different scheduled appointments or did not respond to at least five invitations, one did not cooperate, one had no working phone contacts, and 13 had images with unacceptable technical quality).

Body size and composition

Anthropometrics were obtained with children in underwear and barefoot. Height was measured to the nearest 0.1 cm using a wall stadiometer (SECA, Hamburg, Germany) and weight was measured with a digital scale (Xinyu Electronic Co., Limited, Zhongshan, China) to the nearest 0.1 kg. DXA scans were performed using a Hologic Discovery QDR 4500W device (Hologic Inc., Bedford, Massachusetts), software version 13.3.0.1, while children were barefoot in light indoor clothing and without metal accessories. Total body

less head (subtotal) fat (kg), fat-free mass (kg) and bone mineral content (BMC, kg) were extracted. Lean mass (kg) was obtained by subtracting BMC from fat-free mass.

Socioeconomic position

As socioeconomic position indicators, we selected variables that were both described in the literature as well-documented causes of fat mass [21-23] and supported by empirical observation in our study population [24]. Information was self-reported and collected using face-to-face questionnaires applied to legal guardians in the 7 years of age follow-up with the exception of maternal smoking during pregnancy, which was collected at recruitment. Educational level was recorded as the number of completed years of formal schooling and categorized as higher (>12 years) or less than higher education (≤ 12 years). Occupation was recorded using the main current job and classified according to the International Standard Classification of Occupations (ISCO-08) [25]. Occupations were further grouped into upper white-collar (armed forces, managers, professionals, technicians and associate professionals), and lower white-collar or blue-collar (clerical support workers, services and sales workers, skilled agriculture, forestry and fishery workers, craft and related trades workers, plant and machine operators and elementary occupations). The main caregiver was classified as current smoker (including daily and occasional smokers) or non-smoker (including former smokers with at least six months abstinence and never smokers). Maternal smoking during pregnancy was considered present if mothers had smoked any amount in at least one trimester. Monthly household income was recorded in EUR 500 categories and grouped into two categories: less or equal to EUR 3000 and more than EUR 3000. The type of school that the child attended (public vs. private) was also recorded. Indicators of material living conditions included hiring a housekeeper full or part-time and taking holidays away from home at least once a year.

Data analyses

Figure 2 represents the simplest formulation of our main causal assumptions: if fat mass is a cause of bone mass and SEP is a cause of fat mass, we expect to find a statistical association between SEP and bone mass. We cannot exclude, however, that SEP influences bone mass through a different non-adiposity-mediated mechanism, which explains the direct edge from SEP to bone mass.

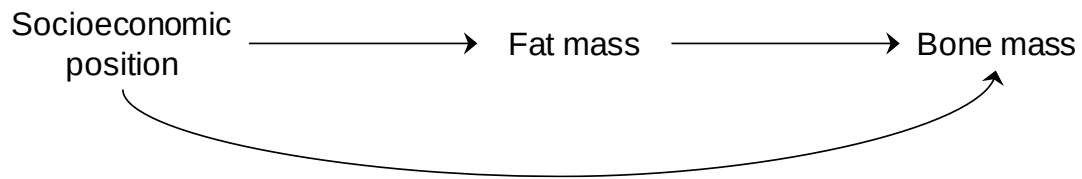


Figure 2. Diagram depicting the causal relations between socioeconomic position (SEP), fat mass and bone mass under the assumptions that: 1) SEP has an effect on fat mass, 2) fat mass has an effect on bone mass, and 3) there is a direct effect of SEP on bone mass not mediated by fat mass. According to the mechanostat theory, the potential direct effect of SEP on bone mass would most likely involve the remaining constitutional components as mediators, summarized in this paper as lean mass and height. When compared to adiposity, the evidence of the effect of SEP on either height or lean mass in younger cohorts is much weaker, even though both of them are documented determinants of bone mass. Therefore, assuming a weak direct (fat-independent) effect of SEP on bone mass, if the magnitude of the fat-mediated effect of SEP on bone is high, we expect that SEP will be statistically associated with bone mass, which would indirectly translate a specific role of adiposity on bone mass

We started our data analysis by estimating the statistical associations between BMC and the remaining body composition and anthropometric variables using linear regression coefficients and their respective 95% confidence intervals (95% CI). This served to test the consistency of Generation XXI data with prior knowledge regarding the fundamental assumption that each body size or composition trait is associated with bone mass. To illustrate the reversal of estimate direction from a crude to a weight-adjusted model documented in previous studies, we calculated a regression coefficient for fat mass adjusted for total body weight. Also using linear regression, we then quantified the magnitude of the associations between each socioeconomic position indicator and height, weight, fat mass, lean mass, and BMC. We used the associations between SEP and fat mass as a positive control and the associations between SEP and height or lean mass as negative controls for our test set of associations, i.e. between SEP and BMC. The term “control” is used here in the laboratory and not the case-control sense, i.e. we expected to find stronger statistical associations between SEP indicators and fat mass than those between SEP and lean mass or, particularly, SEP and height [26]. All anthropometric variables were z-scored using the means and standard-deviations derived from the study sample and regression coefficients are presented crude and standardized to facilitate the comparison of the magnitude of effect estimates.

To estimate the effect on our findings of losses due to nonparticipation and attrition during follow-up, we conducted a sensitivity analysis based on the assumption of a continuous gradient of difficulty in recruitment and retention in cohort studies [27]. When compared to participants who were readily willing to participate in all follow-up waves after a single contact, we assumed that subjects whose participation required more effort on our part were more similar to those who did not participate at all. Therefore, among the children included in our study, we reran a set of analyses by excluding, in separate models, children who did not participate in the 4-years-old evaluation (n=249) and children who underwent the 7-year-old evaluation when they were 7.5 or more (n=158). We also used observed differences between subjects included and those lost to follow-up as an additional marker of participation, and computed estimates after excluding participants who were likely more similar in terms of social background to those lost to follow-up: children whose mothers were younger than 25 years at delivery (n=337) and children whose household crowding index was above 1.5 (n=152). For conciseness we selected three SEP indicators (main caregiver educational level, child's type of school and maternal smoking during pregnancy) and our three main outcomes (fat mass, BMC and height). Statistical analysis was performed with Stata, version 11.2, for Windows (Stata Corp LP).

Results

Our sample of 2408 Generation XXI children (47.4% girls) had mean (SD) age 7.4 (0.4) years, height 124.6 (5.6) cm and weight 27.2 (5.5) kg. The proportions of overweight children were 39.3% and 36.0% according to the WHO and CDC cutoffs, respectively. Mean (SD) subtotal fat mass was 7.7 (3.4) kg and lean mass 14.7 (2.3) kg. Mean (SD) subtotal bone mineral content was 0.60 (0.08) kg. Table 1 reports linear regression coefficients for the associations between each of the body size and composition variables and BMC at 7 years of age. As expected, all variables were clearly associated with BMC and standardized coefficients were lowest for fat mass and highest for height and lean mass. After adjustment for weight, fat mass was negatively associated with BMC.

Table 1. Associations between bone mineral content (g) and body size (weight and height) and body composition (fat mass and lean mass) at 7 years of age (linear regression analysis), Generation XXI, Porto, Portugal (n=2408)

	Change per unit increase ^a		Change per SD increase	
	Crude coefficient	95% CI	Standardized coefficient	95% CI
	(β)		(β_{st})	
Height	0.012	0.011, 0.013	0.069	0.067, 0.071
Total body weight	0.012	0.011, 0.012	0.064	0.062, 0.066
Lean mass	0.030	0.029, 0.031	0.069	0.067, 0.071
Fat mass	0.014	0.013, 0.014	0.047	0.044, 0.050
Fat mass adjusted for weight	-0.021	-0.022, -0.020	-0.072	-0.077, -0.067

^a Height in cm, total body weight, lean mass, fat mass and fat mass adjusted for weight in kg.

Abbreviations: CI, confidence interval; SD, standard deviation

In terms of socioeconomic position indicators (Table 2), more than one third of the main caregivers (the mother in 97.9% of children and the father in 1.4%) and almost half of the secondary caregivers (the father in 89.3% of children and a grandparent in 6.1%) had less than 9 years of formal schooling. Main caregivers were more likely to be white-collar workers while secondary caregivers were frequently upper white-collar workers or skilled blue-collar workers. One fifth of the mothers in our sample had smoked during pregnancy. More than a quarter of children lived in a household with an average monthly

income lower than EUR 1000. Regarding material living conditions, less than one fifth of families hired a housekeeper and one quarter reported not taking holidays away from home.

Table 2. Distribution of participants according to socioeconomic position indicators at 7 years of age, Generation XXI, Porto, Portugal

	n (%) ^a
Main caregiver educational level	
≤9 years	901 (37.5)
10-12 years	749 (31.2)
>12 years	753 (31.3)
Missing	5
Secondary caregiver educational level	
≤9 years	1131 (48.5)
10-12 years	665 (28.5)
>12 years	536 (23.0)
No second caregiver	40
Missing	36
Main caregiver occupation	
Upper white-collar	864 (37.2)
Lower white-collar	977 (42.0)
Skilled blue-collar	247 (10.6)
Unskilled blue-collar	237 (10.2)
Student or Homemaker	4 + 77
Missing	2
Secondary caregiver occupation	
Upper white-collar	970 (41.9)
Lower white-collar	476 (20.6)
Skilled blue-collar	712 (30.8)
Unskilled blue-collar	156 (6.7)
Student or Homemaker	9 + 30
No second caregiver	40
Missing	15
Main caregiver smoking status	
Non-smoker	1860 (77.4)
Current smoker	543 (22.6)
Missing	5
Maternal smoking during pregnancy	
Never smoked	1897 (79.4)

	Ever smoked	491 (20.6)
	Missing	20
Monthly household income		
	≤ EUR 1000	634 (26.8)
	EUR 1001– EUR 3000	1569 (66.4)
	> EUR 3000	160 (6.8)
	Not known or Undisclosed	42 + 2
	Missing	1
Child's type of school		
	Public	2166 (90.2)
	Private	234 (9.8)
	Other	5
	Missing	3
Hiring a housekeeper		
	Full-time	403 (16.8)
	Part-time	33 (1.4)
	No	1968 (81.9)
	Missing	4
Taking holidays away from home		
	Yes	1835 (76.2)
	No	572 (23.8)
	Missing	1

^a Percentages sum 100% for participants included in the analysis of each SEP indicator (n=2403 for main caregiver educational level; n=2332 for secondary caregiver educational level; n=2325 for main caregiver occupation; n=2314 for secondary caregiver occupation; n=2403 for main caregiver smoking status; n=2388 for maternal smoking during pregnancy; n=2363 for monthly household income; n=2400 for child's type of school; n=2404 for hiring a housekeeper; n=2407 for taking holidays away from home).

Figure 3 and supplemental tables 1 and 2 present the magnitudes of the associations between each socioeconomic position indicator and each body size or composition outcome. By design, all 10 SEP indicators selected were associated with fat mass. With the exceptions of monthly household income and child's school type, associations of SEP indicators with total weight showed a very similar pattern to those with fat mass, namely with regard to the rank of magnitudes, with the strongest associations being observed for educational levels of the main caregiver (less than higher education vs. higher education: standardized coefficient (β_{st})=0.29, 95% CI: 0.20, 0.37 for fat mass and β_{st} =0.25, 95% CI: 0.16, 0.33 for total weight) and secondary caregiver (β_{st} =0.28, 95% CI: 0.18, 0.38 for fat mass and β_{st} =0.24, 95% CI: 0.14, 0.33 for total weight). These were followed by main and secondary caregivers' occupations, hiring a housekeeper,

maternal smoking during pregnancy, main caregiver current smoking and taking holidays away from home. With the exception of a comparatively weak association with primary caregiver educational level, height – our negative control – showed negligible associations with all the SEP indicators selected. As for our main outcome, BMC, we found associations with main ($\beta_{st}=0.15$, 95% CI: 0.07, 0.24) and secondary ($\beta_{st}=0.13$, 95% CI: 0.03, 0.22) caregivers' educational levels and main ($\beta_{st}=0.12$, 95% CI: 0.03, 0.20) and secondary ($\beta_{st}=0.10$, 95% CI: 0.01, 0.18) caregivers' occupations. Associations were weaker or imprecise with monthly household income, child's school type, hiring a housekeeper, maternal smoking during pregnancy, taking holidays away from home and main caregiver current smoking. A very similar pattern of associations with SEP indicators was found for lean mass, including the rank and magnitude of standardized measures of association. In addition, for both BMC and lean mass, the magnitudes of the strongest associations with SEP indicators were substantially lower than those found for fat mass and total weight.

Figure 4 presents the results of our sensitivity analysis excluding, in four separate models, children who did not participate in the 4-years-old evaluation, children who underwent the 7-year-old evaluation when they were 7.5 or more, children whose mothers were younger than 25 years at delivery and children whose household crowding index was above 1.5. In each of these four subsamples we found similar associations between SEP indicators and fat mass, BMC and height to those found for the whole sample.

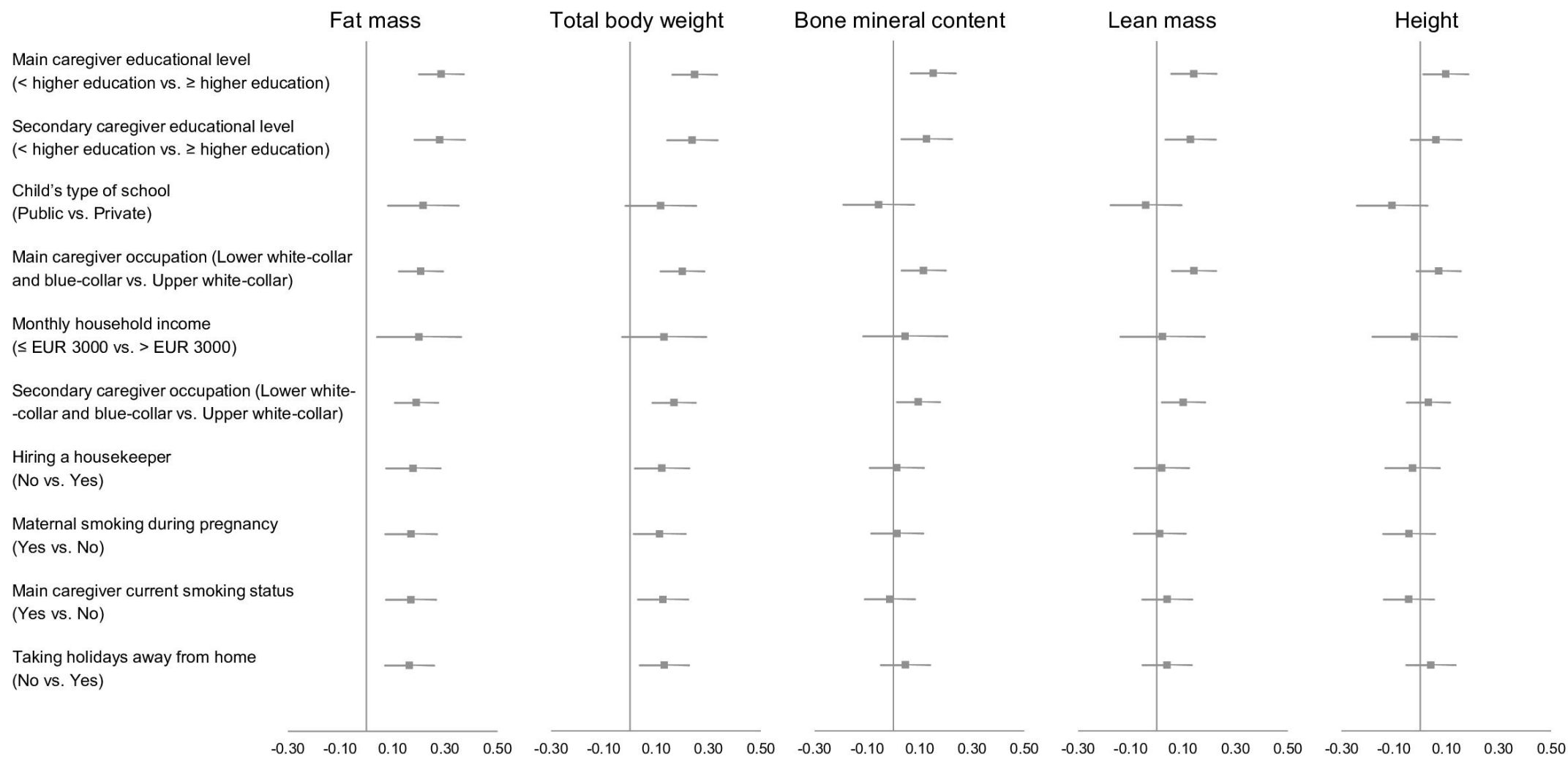


Figure 3. Standardized linear regression coefficients (β_{st}) and 95% confidence intervals for the associations between socioeconomic position indicators and each of the body size and composition outcomes. Socioeconomic indicators ordered by decreasing strength of association with fat mass

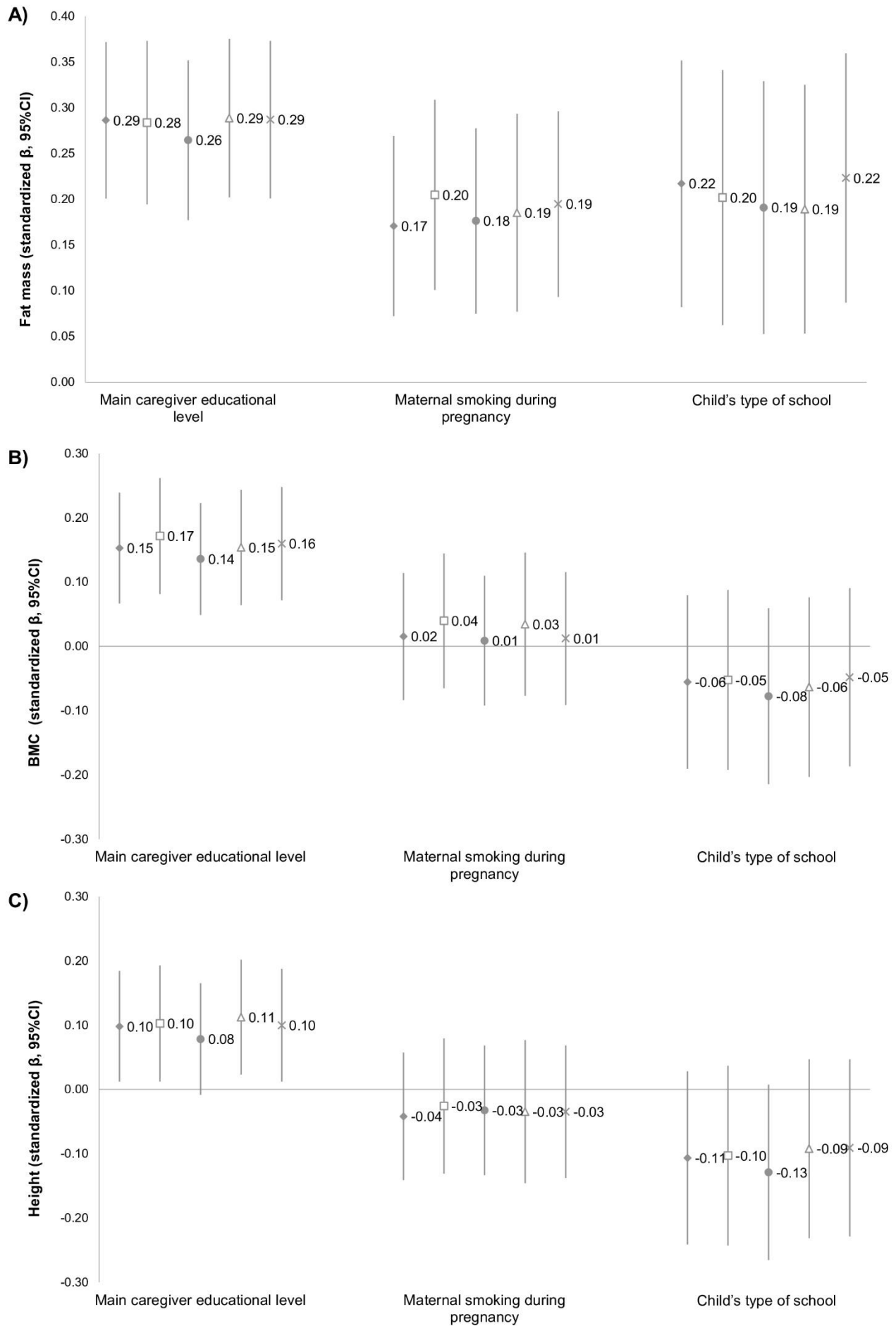


Figure 4. Standardized linear regression coefficients (β) and 95% confidence intervals for the associations between socioeconomic position indicators (main caregiver educational level, child's type of school and maternal smoking during pregnancy) and body size and composition outcomes - A) Fat mass, B) BMC and C) Height.

Diamond: whole sample of children included in the analysis of each socioeconomic position indicator (n=2403 for main caregiver educational level; n=2388 for maternal smoking during pregnancy; n=2400 for child's type of school). Square: excluding children who did not participate in the 4-years-old evaluation (n=2156 for main caregiver educational level; n=2142 for maternal smoking during pregnancy; n=2153 for child's type of school). Circle: excluding children who underwent the 7-year-old evaluation when they were 7.5 or more (n=2246 for main caregiver educational level; n=2232 for maternal smoking during pregnancy; n=2242 for child's type of school). Triangle: excluding children whose mothers were younger than 25 years at delivery (n=2066 for main caregiver educational level; n=2056 for maternal smoking during pregnancy; n=2063 for child's type of school). Cross: excluding children whose household crowding index was above 1.5 (n=2252 for main caregiver educational level; n=2237 for maternal smoking during pregnancy; n=2248 for child's type of school). BMC, bone mineral content.

Discussion

In this work our central assumption was that traits that are causally related share causes to a measurable extent. We found that socioeconomic predictors of fat mass at 7 years of age were in general weakly associated with bone mineral content at the same age. Our causal interpretation is that there is little evidence of a specific effect of adiposity on bone mass in 7-year-old children beyond its contribution to body size.

We decided to address this issue due to the singularity of the relation between adiposity and bone resistance. The observation that fat mass has a crude positive association with bone mass is generalized, but a causal interpretation of those findings would have delicate implications in the face of the numerous harms of obesity. This is no reason to discredit a beneficial effect of fat on bone, but it is certainly a reason to assess its robustness. A number of papers have attempted to isolate the effect of fat by conditioning on the remaining body size and composition measures, and many found an inversion of adjusted estimates (also illustrated in our results) that would suggest a harmful effect of fat on bone [28]. However, this issue is particularly challenging to study using anthropometric and body composition traits alone, since the different measures are very closely related, physiologically and constitutionally, particularly during growth. In practice, it is very likely that any measure of body size or composition is statistically associated with bone mass simply because it is part of the same organ system or because it evolves together with the skeleton during growth. In fact, height velocity drives lean mass, fat mass, and bone mass velocities, all of which peak within a 10-month period [29]. In the present study, this is demonstrated in the strong associations of height, weight, fat mass and lean mass with bone mineral content. This analytical problem is also illustrated by the implausibility of directed acyclic graphs that attempt to represent relations between traits whose expression is largely regulated by physiological feedback. The common endocrine control of body composition during growth renders directional causal assumptions particularly challenging to formulate or test and may explain the inversion of adjusted estimates through collider and/or sparse data biases [16,30].

We believe that the population health perspective requires analytical approaches that address causation without overworking empirical data, while taking advantage of a special attention to different disease causation levels. Therefore, we chose a benchmark from outside the domain of physiology, but with documented specificity for fat, i.e. more strongly causally related with fat than with other body size or composition traits. We assumed that if there was a strong causal relation between fat mass and bone mineral

content then the causes of fat mass would also be indirect causes of bone mass, to an extent that we could estimate using statistical associations. In this framework, our main finding was the difference between fat mass and bone mass in terms of their associations with SEP indicators. It is well-known that social background is related with adiposity in high-income countries [18,22,31]. While this was replicated in our study, we now add that the same ten SEP indicators in the same cohort have much weaker or negligible associations with bone mineral content. However, we acknowledge that the observed association between SEP and bone mass may be the net result of two different components, only one of which mediated by fat (Figure 2). The present results may be due to the cancelling out of a fat-mediated effect by a direct effect of SEP on bone in the opposite direction. This has been suggested in another birth cohort, where authors concluded that social position seemed positively related to bone mass due to increased height but that this influence was counteracted by a positive effect of fat deposition on bone area [32]. Although we cannot exclude this possibility, a height-mediated effect of SEP on bone is not likely in our population, since little evidence of an effect of SEP on height was found in the whole cohort [26]. The explanation might be that Generation XXI children were born more recently (2005-2006) and socioeconomic disparities in height have narrowed substantially over the last decades in several contexts [18,33,34]. In fact, we took advantage of this absence of a measurable effect of SEP on height to use it as a negative control for our findings regarding adiposity. An additional relevant aspect that we did not address here is confounding by parental anthropometry: a large genetic study found that one's height and BMI are likely to have an effect on one's SEP, which implies that parental anthropometry could be a common cause of parental SEP and child anthropometry in our study [35]. However, if this was a major source of confounding in the younger generations, we would again expect to find an association between parental SEP and children's height, which we did not.

Interestingly, the pattern of associations with SEP was very similar between bone mineral content and lean mass, with caregivers' educational level and occupation as the only potential causes. This is consistent with previous findings of high correlations between muscle and bone mass in children, which are typically stronger than those between fat and bone [36]. The interpretation might be that lean mass determines bone mass or, even more likely, both are a result of common environmental exposures, such as physical activity, that may modulate overall musculoskeletal development while being influenced by particular literacy-related features of SEP such as educational level. Nevertheless, we acknowledge that the similarity between bone and lean mass

outcomes might be inflated due to the fact that both were extracted from the same whole-body DXA scan.

Socioeconomic position is a complex construct and its measurement challenging. By design, we chose to test only SEP indicators that were associated with adiposity, leaving out other potential measures that were not predictive of fat mass in our target population. We recognize that this option may have disregarded important dimensions, although we believe we have captured the most relevant SEP indicators in our context [24]. In addition, SEP was used here to establish a positive benchmark due to its association with adiposity. The inclusion of indicators that are not associated with fat mass would not contribute useful information to test our main hypothesis. Also with regard to exposure assessment, we cannot exclude that SEP indicators collected using a face-to-face questionnaire were subject to social desirability or unwillingness to disclose, but it seems unlikely that this has had a large impact on our findings since they were robust to 10 different SEP indicators and to different exposure categorization strategies (latter results not shown). Our use of dichotomous variables for SEP indicators also likely diluted the effect of misclassification between finer categories, which were not essential to test our hypothesis.

Several voluntary and involuntary filters sequentially affected the composition of the final sample. We imposed a design filter on sample size by consecutively selecting a subsample of around 3000 children for DXA scans and used a technical quality criterion for scans. We believe these options had negligible effects on our estimates, since the former was solely based on equipment availability and funding schedule and the latter affected a small number of exams. More importantly, however, the results presented here were obtained in individuals whose families accepted to participate in the cohort study, to be followed up to age seven, to undergo DXA, and to report on SEP indicators. A number of different mechanisms may be hypothesized to explain nonparticipation, losses to follow-up or unwillingness to disclose particular information. To assess their impact on our findings, we opted to conduct a sensitivity analysis based on the assumption that participant-dependent filters (nonparticipation, attrition and nondisclosure of information) act probabilistically [27]. We considered that, among participants, there are individuals who are more similar to extreme nonparticipants and that this similarity may be assessed on the basis of their record of interactions with the research team and background characteristics. Our sensitivity analyses showed that exclusions had a minor impact on our main findings, suggesting that our results were not seriously biased in this regard.

It is possible that our findings are not generalizable to older birth cohorts, where the relations between socioeconomic background and body size seem at least quantitatively different. We can also hypothesize different causal structures in other populations where our results would not necessarily be applicable. However, as long as SEP is specific for adiposity when compared to other body size and composition traits, we believe that our findings may be replicable in other settings. An important external validation is that our results on the effect of SEP on bone quality generally agree with those of previous studies in high-income countries [32,37-40]. Finally, our study does not intend to provide estimates for children with established metabolic or hormonal disease, even though the present findings were obtained from a sample where more than one third of children were overweight at age seven, accurately reflecting the source population.

In conclusion, in a setting where SEP is an undisputed cause of adiposity, we found little evidence of a social determination of bone mineral content at 7 years of age. Our interpretation is that either fat mass does not have a specific effect on bone beyond its contribution to body size or that it has a small comparative effect, positive or negative, not enough to overcome that of other constitutional traits. The main implication of our study is that adiposity is unlikely to be an important cause of bone mass in childhood at the population level.

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Supplemental table 1. Linear regression coefficients (β) and 95% confidence intervals for the associations between socioeconomic position indicators and each of the body size and composition outcomes

	Fat mass (kg)			Total body weight (kg)			Bone mineral content (kg)			Lean mass (kg)			Height (cm)		
	β	95% CI		β	95% CI		β	95% CI		β	95% CI		β	95% CI	
Main caregiver educational level (less than higher education vs. higher education)	0.99	0.69	1.28	1.37	0.90	1.84	0.01	0.01	0.02	0.33	0.13	0.53	0.55	0.07	1.03
Secondary caregiver educational level (less than higher education vs. higher education)	0.97	0.63	1.30	1.32	0.79	1.85	0.01	0.00	0.02	0.30	0.08	0.52	0.34	-0.20	0.88
Child's type of school (Public vs. Private)	0.75	0.28	1.21	0.65	-0.09	1.40	0.00	-0.02	0.01	-0.09	-0.41	0.22	-0.59	-1.34	0.16
Main caregiver occupation (Lower white-collar and blue-collar vs. Upper white-collar)	0.72	0.43	1.01	1.11	0.64	1.57	0.01	0.00	0.02	0.33	0.13	0.52	0.39	-0.07	0.86
Monthly household income ($\leq$ EUR 3000 vs. $>$ EUR 3000)	0.69	0.14	1.25	0.72	-0.16	1.61	0.00	-0.01	0.02	0.05	-0.32	0.42	-0.12	-1.01	0.78
Secondary caregiver occupation (Lower white-collar and blue-collar vs. Upper white-collar)	0.66	0.37	0.94	0.93	0.48	1.39	0.01	0.00	0.02	0.24	0.05	0.43	0.18	-0.28	0.64
Hiring a housekeeper (No vs. Yes)	0.62	0.26	0.97	0.68	0.11	1.25	0.00	-0.01	0.01	0.05	-0.19	0.28	-0.16	-0.74	0.42
Maternal smoking during pregnancy (Yes vs. No)	0.59	0.25	0.93	0.63	0.08	1.17	0.00	-0.01	0.01	0.03	-0.20	0.26	-0.23	-0.78	0.32
Main caregiver current smoking status (Yes vs. No)	0.59	0.26	0.92	0.70	0.17	1.23	0.00	-0.01	0.01	0.09	-0.13	0.31	-0.24	-0.77	0.30
Holidays away from home (No vs. Yes)	0.57	0.24	0.89	0.73	0.21	1.24	0.00	0.00	0.01	0.09	-0.12	0.31	0.23	-0.29	0.75

Socioeconomic indicators ordered by decreasing strength of association with fat mass.

Supplemental table 2. Standardized linear regression coefficients (β_{st}) and 95% confidence intervals for the associations between socioeconomic position indicators and each of the body size and composition outcomes

	Fat mass (z-score)			Total body weight (z-score)			Bone mineral content (z-score)			Lean mass (z-score)			Height (z-score)		
	β_{st}	95% CI		β_{st}	95% CI		β_{st}	95% CI		β_{st}	95% CI		β_{st}	95% CI	
Main caregiver educational level (less than higher education vs. higher education)	0.29	0.20	0.37	0.25	0.16	0.33	0.15	0.07	0.24	0.14	0.06	0.23	0.10	0.01	0.18
Secondary caregiver educational level (less than higher education vs. higher education)	0.28	0.18	0.38	0.24	0.14	0.33	0.13	0.03	0.22	0.13	0.03	0.23	0.06	-0.04	0.16
Child's type of school (Public vs. Private)	0.22	0.08	0.35	0.12	-0.02	0.25	-0.06	-0.19	0.08	-0.04	-0.18	0.09	-0.11	-0.24	0.03
Main caregiver occupation (Lower white-collar and blue-collar vs. Upper white-collar)	0.21	0.12	0.29	0.20	0.12	0.28	0.12	0.03	0.20	0.14	0.06	0.23	0.07	-0.01	0.15
Monthly household income ($\leq$ EUR 3000 vs. $>$ EUR 3000)	0.20	0.04	0.36	0.13	-0.03	0.29	0.05	-0.11	0.21	0.02	-0.14	0.18	-0.02	-0.18	0.14
Secondary caregiver occupation (Lower white-collar and blue-collar vs. Upper white-collar)	0.19	0.11	0.27	0.17	0.09	0.25	0.10	0.01	0.18	0.10	0.02	0.18	0.03	-0.05	0.11
Hiring a housekeeper (No vs. Yes)	0.18	0.08	0.28	0.12	0.02	0.23	0.01	-0.09	0.12	0.02	-0.08	0.12	-0.03	-0.13	0.08
Maternal smoking during pregnancy (Yes vs. No)	0.17	0.07	0.27	0.11	0.02	0.21	0.02	-0.08	0.11	0.01	-0.09	0.11	-0.04	-0.14	0.06
Main caregiver current smoking status (Yes vs. No)	0.17	0.07	0.27	0.13	0.03	0.22	-0.01	-0.11	0.08	0.04	-0.06	0.14	-0.04	-0.14	0.05
Holidays away from home (No vs. Yes)	0.16	0.07	0.26	0.13	0.04	0.23	0.05	-0.05	0.14	0.04	-0.05	0.13	0.04	-0.05	0.14

Socioeconomic indicators ordered by decreasing strength of association with fat mass.

5. Overall discussion

5.1. Main findings

In a large population-based prospective cohort study of children and their mothers, we explored the effects of growth during different stages of the life course on bone mineralization at 7 years of age.

Beginning in the intrauterine life, our results were indicative of heterogeneous associations between weight gain by mothers during pregnancy and offspring bone mineralization, according to maternal body mass index in early pregnancy. Among women who were under/normal weight at the beginning of pregnancy, gestational weight gain was slightly associated with increased offspring bone mineralization at 7 years, while in overweight/obese mothers increased gestational weight gain had no effect on childhood bone measures. When we analyzed gestational weight gain by using Institute of Medicine recommended categories for healthy weight gain during pregnancy, we found that excessive weight during pregnancy had no overall advantageous effect on childhood bone mineralization in both under/normal weight and overweight/obese mothers.

As for the role of children's growth in their bone mineralization at 7 years of age our findings suggested that both height and weight velocities in the first 6 years of life were clearly associated with increased childhood bone mass. Growth between 1 and 3 years had the strongest positive associations with childhood bone mineral mass, a finding possibly attributable to the adoption of the upright position and the development of walking abilities. Despite a strong statistical dependence on body size, this finding held for both height and weight growth. In addition to the identification of early childhood as a particularly sensitive period for the effect of growth on bone development, we found that differences in the overall shape of growth since birth up to the age of six, particularly with regard to the trajectory of weight gain, were also relevant for childhood bone mass and density. Specifically, children who followed trajectories of increased weight gain since birth, reflecting greater and longer exposure to loading, had clearly increased bone mineralization at the age of seven. Adjustment for concurrent body size uncovered the strong biological and statistical dependence between growth and the final weight attained, while suggesting a beneficial effect of a trajectory of later weight gain in comparison to an average (normal) trajectory. However, these important effects of early life growth on bone mineralization were not observable when we analyzed measures of bone turnover. In fact, in contrast to what happens in the clinical pediatric setting, bone formation and resorption markers seem to have limited utility in generally healthy children, specifically for describing overall anthropometric growth and bone mineralization status.

With respect to the relative contribution of components of weight, our results suggested that lean mass was a more important determinant of childhood bone mineralization than fat mass. We further explored the potential of fat mass for specifically affecting childhood bone mass through a singular approach that used common causes as a benchmark, i.e. assuming that traits that are causally related share causes to a measurable extent. In view of the absence of social determination of bone mass, in a context where fat mass is clearly determined by socioeconomic position, we suggest that fat mass may not have a specific effect on bone mass, beyond its contribution to childhood body size, or its effect is small in comparison to other body size and composition traits.

In this thesis, despite different underlying research questions in the two studies assessing the role of infancy and childhood growth on bone mineralization, the results from both studies showed consistent strong positive associations between growth and bone. The fact that there was a broad agreement in the results between these approaches, despite different underlying assumptions, further reinforces our conclusion that growth is an important determinant of bone mineral accrual. Even though results from studies using distinct statistical approaches to model growth are not always straightforwardly comparable, the different methods of analysis are complementary and it is recommended to use more than one approach to describe growth associations [138].

Additionally, although we recognize that maternal gestational weight gain reflects increases in several traits other than fetal growth, our findings of gestational weight gain and bone associations also contribute to show the relevance of growth, from as early as the intrauterine life, as a determining factor of childhood bone mineralization [119].

An additional issue in our results is that we steadily found more consistent and strong associations of growth with bone mineral content (the most size-dependent measure) and much less marked associations with bone density. These findings are not surprising as they reflect the clear interdependency between the size of the overall skeleton and whole body growth, both in height and in weight.

5.2. Implications for clinical practice and policy

Throughout the thesis, potential implications of our findings at the policy and clinical practice levels are presented. However, we must recognize that etiological research does not aim to test the effectiveness of recommendations and other policy changes, and may not be directly applicable to the clinical and policy settings. If implications are

to be extracted, however, at the individual level, our findings support the inclusion of children's skeletal health as a goal for weight counseling during pregnancy. Women should be informed that adherence to weight gain recommendations during pregnancy will also contribute to optimize their child's skeletal health. Additionally, our results point to the importance of identifying disturbances in normal growth, particularly during early childhood, that may impact bone mineralization. Regarding the serum measurement of bone metabolism markers, our results do not support the general implementation of these measurements as part of standard clinical care of healthy children to monitor the status of bone mineral accrual. At the policy level, our findings reinforce the application of the Institute of Medicine guidelines for weight gain during pregnancy in order to also promote child skeletal health [122]. Furthermore, this thesis provided some evidence to highlight that strategies to optimize childhood growth before puberty are likely to have beneficial effects on bone mineralization. Specifically, interventions aiming to optimize childhood bone health might be more successful in early childhood. Additionally, preventive strategies or interventions aiming at reducing childhood adiposity might not be particularly relevant to promote childhood bone mineral accrual.

5.3. Methodological considerations

5.3.1. Selection bias

In the present work, we analyzed data from a cohort of children followed prospectively since birth, and evaluated at 4 and 7 years of age. Selection bias at recruitment is possible, since only women giving birth in public maternities were invited to participate, therefore excluding women delivering in private facilities. Although it was estimated that, at the time of recruitment, births occurring in public hospitals corresponded to 91.6% of the total number of deliveries occurring in the whole catchment population, an overrepresentation of women from lower socioeconomic background and of those with complicated pregnancies is expected. In the Porto region, in accordance with national practice, level III public maternity units with differentiated pre and perinatal support are the preferential referral centers for high risk pregnancy cases which are also at greater risk of obstetric complications. This may mean that women with complicated pregnancies or who were not willing or capable to pay for private health care will be overrepresented in public maternities. An additional methodological decision, and potential source of selection bias, was the fact that not all eligible mothers were invited to participate in the cohort study because of logistic constraints, namely availability of human resources. In these circumstances, 70% of the eligible mothers were invited on a first come first served

basis [187]. To understand the impact of these selection options on potential selection bias, comparisons regarding main demographic and delivery characteristics between cohort participants and all women delivering in the catchment area and in Portugal were previously conducted using official statistics published by Statistics Portugal [188]. In comparison to women from the catchment area who gave birth between 2005 and 2006, mothers that integrate the cohort were slightly younger although similar in what regards to marital status. The prevalence of caesarean section in the cohort was slightly higher than the prevalence estimated for Portugal in the year 2004. Regarding delivery characteristics, a similar sex distribution was observed between babies included in the cohort and all babies born in Portugal in the corresponding period. However, a higher proportion of multiple fetuses was found in the cohort participants compared to national estimates. Also, in comparison to deliveries in Portugal between 2005 and 2006, the cohort was characterized by a higher proportion of preterm and low birth weight newborns, which might be reflecting the overrepresentation of complicated pregnancies in public maternities with differentiated perinatal support. It is difficult to speculate whether these differences may have biased our effect estimates. However, since the selection of public maternities was not related to the objectives of this thesis, we expect that these selection criteria have not largely biased our associations. An additional selection strategy that needs to be discussed as potential source of selection bias is that related to the subset of participants included in our analyses. These participants represented a consecutive sample of all children evaluated in-person at 7 years of age, who were invited to perform a DXA scan. These participants corresponded to 27.8% of the entire cohort who differed from the remaining cohort participants in anthropometric and sociodemographic characteristics. In view of the minor magnitude of these differences and, again, of the independence of sample selection criteria from the objectives of this study, we expect minor selection bias.

5.3.2. Participation bias

An additional issue that needs to be assessed in cohort studies is the extension of potential participation bias, which may occur due to nonparticipation at baseline or attrition throughout follow-up. In the Generation XXI study, 91.4% of the invited mothers accepted to participate. Mothers who refused to participate are likely to be systematically different from those who accepted to take part in the evaluations. However, owing to ethical issues it was not possible to collect information on eligible individuals who refused to participate and therefore to estimate the scope of such differences. Nevertheless, the high participation proportion at baseline may decrease the impact of that potential

limitation [180]. We also believe, as previously demonstrated in another cohort study, that our results were not strongly biased by nonparticipation at baseline [189]. Regarding losses to follow-up, our analyses used data collected at the 4 and 7-years-old reevaluations, in which 69.2% and 67.6% of the entire cohort, respectively, participated in the in-person evaluation. These attrition rates of around 30% may not have been an important source of bias in the results of this thesis, particularly in results from regression analyses, since a previous study suggested that, even when subjects who stay differ from those who drop out of a study, estimates of associations tend to remain robust to such differences [190]. In each of the present studies, comparisons between children and mothers who were and were not included in the analyses revealed differences in sociodemographic and anthropometric characteristics collected at recruitment and during follow-ups. The magnitude of those differences was however generally small. This observation supports our belief that our effect estimates were not largely biased due to selective loss to follow-up. Additionally, whenever possible, our analyses were adjusted to factors that were presumed to affect censoring and outcomes simultaneously [141]. Specifically in paper I, bearing in mind that in our setting, the most important determinants of nonparticipation at baseline and attrition throughout follow-up were in fact determinants of misclassification of pregnancy weights, our sensitivity analyses, which revealed a minor impact of weight misclassification, also suggests that our estimates are not substantially biased by attrition and nonparticipation. In each study, we opted for complete case analysis, which assumes that the probability of being a complete case is independent of the outcome variable, i.e. the bone mineral properties attained, after adjustment for all the important covariates [191]. Overall, although differential losses to follow-up are a major concern for the generalizability of findings from birth cohorts, these studies remain the most valuable approach to assess associations between risk or protective factors and later health outcomes [191]. Cohort studies are particularly useful to understand the temporal sequence between exposure and outcome, to estimate effects in a comparatively long-term, and to study trajectories of growth during infancy unaffected by cohort or period effects [192].

5.3.3. Misclassification

Regarding potential misclassification, we believe that differential misclassification of exposures was not a significant problem in our studies. Data on exposures was collected longitudinally, before the assessment of bone outcomes, and the team of technicians that performed DXA scans to obtain bone measurements was blinded to the exposure status. Both interviewers and parents or legal guardians were unaware of the specific

research questions under study. Nevertheless, non-differential misclassification, a source of random error, might have reduced our statistical power and/or induced biased results. Information on prepregnancy and predelivery maternal weight was self-reported and an underreporting of weight associated to pregnancy has been previously described [193]. However, misreporting weight might not be a significant source of bias in associations between gestational weight gain and childhood bone outcomes as previously demonstrated for other offspring outcomes [194]. We must also acknowledge that anthropometric measurements used in growth modeling were obtained from health care records, which included the contribution of several health professionals with different measurement equipment. Height/length and weight data routinely collected from child health records has previously shown to have little systematic error [195] but we cannot rule out the possibility of random error.

Regarding the possible misclassification of bone outcomes, it is important to recognize some limitations of DXA. The main disadvantage of DXA is its two-dimensional methodology of a 3D structure that results in an estimate of BMD that is not a true material volumetric density but an areal measure easily influenced by bone size [61]. The impossibility of DXA to account for the third dimension leads to an erroneous estimation of aBMD i.e., considering two bones with the same volumetric density, the large bone will be assigned a higher value of areal density than the smaller one. This makes BMD assessment in growing children particularly challenging. Many statistical procedures have been proposed to decrease the influence of bone size on bone mass and density measures and these go from the inclusion of bone area, weight and height as independent variables in multiple regression models to height adjustment equations [61,182]. In the present thesis, we opted to compute a measure of size-corrected BMC (scBMC) to account for bone size effects. This measure was derived by linear regression of BMC on bone area and addition of the resulting residuals to the mean sample's BMC [182]. This was postulated as an adequate correction of BMC for skeletal size differences and as an approximate measure of volumetric BMD [196]. Alternatively, in order to clarify the size dependency of bone mass, we used as height at age 7, as a measure of linear growth, and BMC adjusted to height, as a measure of bone mass relative to body size, as outcomes. The latter outcome aimed to provide an estimate of skeletal growth relative to height up to that point, more closely related to cumulative bone growth than to the physical properties of bone tissue. Additionally, we also performed analysis using BMC and areal BMD without size adjustment. We acknowledge that these outcomes are biased by current body size, and are not accurate measures of the material properties of bone tissue. However, taking into account that both material density and bone size

contribute to overall bone strength, BMC and areal BMD also reflect overall bone strength [197]. This is also supported by the accuracy of BMC to predict the risk of fracture in children, which is ultimately a key clinical validation [61].

Another important limitation of DXA is that it cannot distinguish trabecular from cortical bone. In our work, this distinction could have helped to clarify the relationships between bone metabolism markers and bone mineral properties, since trabecular bone is considerably more available for metabolic processes than cortical bone [145]. To overcome this limitation we analyzed bone outcomes at the lumbar spine as a proxy of trabecular bone, since this anatomical site is mostly composed of this type of bone.

Overall, we opted to use DXA to estimate bone strength because this is the recommended method for assessing BMC and BMD in children due to safety, rapid scan times and low radiation, which are especially important features in studies conducted in children [61,198]. Additionally, the fact that DXA is the most widely used method for bone assessment allows for comparisons between settings and populations. We followed the recommendations for research in pediatric populations of the International Society for Clinical Densitometry and we analyzed total body less head (subtotal) bone measures [61]. The exclusion of the head is recommended because skull mineralization is not responsive to physical or environmental stimuli, it accounts for a large percentage of the skeleton, and skull fractures do not represent fragility fractures. To avoid additional exposure to radiation, we did not collect data on reproducibility using repeated measurements for each subject, which would have been essential to quantify *in vivo* precision. However, reproducibility of the DXA device was tested using the spine phantom with and without repositioning, in two distinct moments, and the estimated coefficient of variation was below 1%.

5.3.4. Confounding

To account for confounding, all analyses were adjusted for potential common causes of exposures and outcomes. We selected covariates based on the literature or on their associations with the exposures and outcomes of interest. In general, results remained practically unchanged after adjustment for potential confounders, which advocates in favor of the robustness of our findings. Although information about many potential confounders was available in the studies included in this thesis, residual confounding might still be present. Additionally, the self-reported nature of several confounders might have contributed to residual confounding.

An important concern regarding our findings, is whether associations of maternal weight gain during pregnancy with childhood outcomes reflect direct intrauterine mechanisms, i.e. programming, or confounding by shared environmental, lifestyle or genetic characteristics as previously suggested for other outcomes such as childhood obesity [199] or, more likely, a cumulative effect of these two. Likewise, relationships between longitudinal growth and later bone mineralization might be, at least partially, explained by the overlapping genetic determination of these traits or the common endocrine control of body composition during growth, which is also, to a large extent, genetically determined [88]. Nevertheless, the identification of genetic or hormonal determinants of growth and bone associations are outside the scope of this work. A key consideration is the observational nature of our studies that raises important challenges to infer effects of gestational weight gain, growth or body composition traits on bone mineralization. These influences should be seen in the context of homeostatic feedback mechanisms rather than as a set of unidirectional effects, which has particularly challenging implications regarding the control for confounding.

5.4. Interpretation of main findings

Although the results of this thesis provided some evidence that early life growth impacts subsequent bone development in childhood, the extent to which such an influence could persist up to peak bone mass and beyond to decrease the risk of fragility fracture in adulthood is unclear [200,201]. Evidence of bone mass tracking in longitudinal studies is relatively short-term and its implications are contingent on the assumption of the peak bone mass model. It is undeniable that childhood is an important period for the attainment of bone strength because of the great amount of bone mineral acquired during this stage (Figure 18, A). Therefore, it is widely assumed that interventions aiming at increasing bone mass acquisition in childhood will optimize PBM and may have a persistent effect on bone mass throughout life and ultimately decrease fragility fracture risk (Figure 18, B) [83]. However, one of the main limitations of the concept of optimizing PBM is that it is unidimensional and may oversimplify the complex dynamics of bone development during growth [201]. If bone mass is governed by a homeostatic system acting in the short-term with any perturbation tending to be corrected over time (as defended by the mechanostat theory), bone mass may depend primarily on recent conditions rather than on the distant past [124]. This would mean that the effect of an intervention could disappear over time as bone mass tends to revert to a homeostatic set point (Figure 18, C, D, F). In view of this, optimizing PBM may not have a relevant benefit on fracture occurrence. Indeed, laboratory findings and clinical trials have raised

doubts regarding the effectiveness of interventions of increasing PBM (Figure 18, C) and of increasing PBM to maintain higher bone strength throughout adulthood (Figure 18, D).

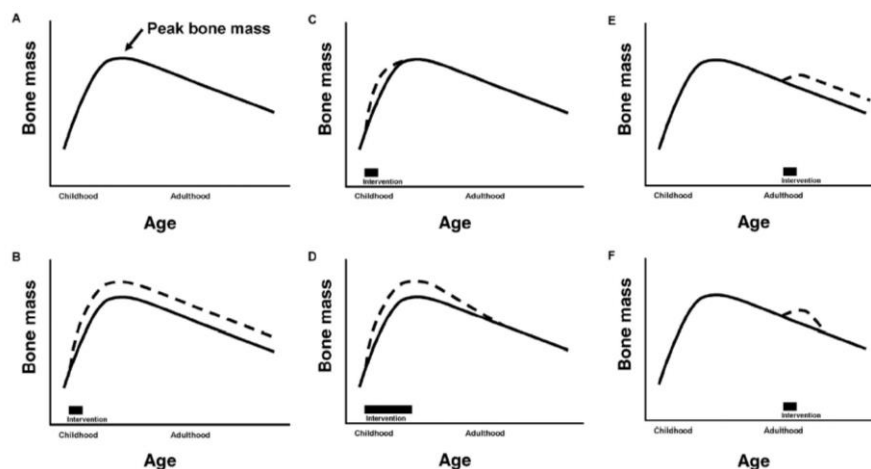


Figure 18. Proposed models for the effect of interventions to increase peak bone mass. The dashed curves represent the bone mass resulting from the intervention; the solid curves represent bone mass in the absence of intervention (reproduced from Gafni & Baron [200])

However, even if optimizing bone mineral accrual by intervening on modifiable factors does not improve PBM or if PBM optimization by itself does not decrease fracture risk, it is very likely that modifiable determinants of bone properties, such as body size and composition, are similar since intrauterine life until adulthood. Additionally, pregnancy and childhood are critical for establishing health-related behaviors affecting maternal gestational weight gain and childhood growth. These behaviors, particularly related with nutrition and physical activity are likely to be shared by mothers and children, and persist throughout the life course [202-204]. As such, even if there are no direct long-term effects on adult bone from interventions on modifiable determinants of healthy maternal weight gain and childhood growth, early intervention on these life-long causes of bone fragility may have indirect persistent effects, if such exposures keep their relevance throughout the life course. As such, the search for the etiology of bone fragility from early life and the intervention upon its modifiable causes seems to remain relevant as a life course approach, which could result in an early initiation of sustained beneficial health related-behaviors rather than a “one-time investment” in bone quality with long-term effects.

We also acknowledge that, from a biomechanical point of view, bone mass changes have limitation in describing peak bone strength attainment. Indeed, bone growth cannot strictly be described as an increase in the material density of existing tissue [205], but rather as a redistribution of bone tissue that causes an increase in mass in areas where it optimizes bone strength for the least possible mass [201]. This is the prevailing view

during the reproductive period, where no adaptive advantage of overmineralized bones is likely. However, it is possible that in the generally healthy pediatric population the full genetic potential is defined as the most efficiently adapted skeleton in terms of amount and distribution of bone mass. Differences in bone mass between children of similar body size may be seen as a measure of the attainment of that genetic potential, which, although arguable, is one of the main premises of the present thesis.

The Generation XXI birth cohort has allowed for the collection and analysis of a wide spectrum of data related to several dimensions of childhood health since intrauterine life. Specifically regarding bone health, the Generation XXI has provided a valuable framework to measure and characterize physical and biochemical measures of bone quality, as well to test potential effects of prenatal determinants and longitudinal growth on childhood bone health.

The fact that all children were born in a short period of 17 months, between 2005 and 2006, and were reevaluated at the same age minimized possible confounding by age, cohort or period effects. However, as a representation of the Portuguese context, the Generation XXI population is comparatively homogeneous in terms of geographical origin, which may limit the generalizability of our results to other settings. Nevertheless, the population-based design of the Generation XXI cohort optimizes the generalizability of findings, potentially extending their applicability within the public health framework, namely to design population-targeted interventions that are potentially more effective and efficient in the general population.

Regarding the timing of bone measures, we should note that Generation XXI is a multi-purpose cohort designed to identify effects of numerous exposures on several dimensions of health. We decided to perform bone densitometry close to the 7 years-old follow-up evaluation because this age represents a biological milestone before the onset of puberty, when sexual development becomes a major driving force for growth and development, particularly regarding micro and macroarchitectural changes to the skeleton. In a life course approach to the study of the etiology of suboptimal bone strength, the prepubertal years represent an important milestone to identify and intervene in environmental determinants of bone mass.

5.5. Future research

A number of major issues remain to be addressed in future studies. In terms of etiology, it will be relevant to assess whether these findings have long-lasting repercussions on the children's bone mass as they go through adolescence and enter adulthood. In a more distant future, it will be important to find out whether maternal pregnancy weight gain, and early life growth and body composition influence the most relevant long-term clinical outcome, i.e. fragility fracture risk in older ages. As for policy, we still have a long way to go in terms of knowing the real-world effectiveness of specific interventions namely to improve adherence to weight gain recommendations. Both pregnancy and childhood may be a critical opportunity in humans' lives to build up or reinforce health promotion skills and literacy, but pragmatic evidence from public health research is still needed. Given the inherent limitations of observational studies, particularly regarding the study of physiological mechanisms, evidence obtained from basic molecular biology, genetic epidemiology, instrumental variable approaches, natural experiments and randomized controlled trials may be relevant to complement our findings [82].

6. Conclusions

In the present thesis, we found no beneficial effect of excessive maternal gestational weight gain on childhood bone mineralization, in both normal weight and overweight women prior to pregnancy, from which we concluded that adherence to the current recommendations for weight gain during pregnancy will contribute to optimize child's skeletal health.

With regard to effects of postnatal growth on bone development, weight and height velocities in the second and third years of life had the largest impact on childhood bone measures suggesting that early childhood represents a sensitive period for the effect of growth on childhood bone mineralization. Additionally, the overall trajectory of weight since birth may shape the development of childhood bone mineralization. The overall growth trajectory was not, however, particularly associated with childhood bone metabolism markers, which may represent more dynamic measures of bone turnover. Those markers were also negligibly associated with bone mineralization outcomes suggesting a limited ability to describe overall growth and bone mass in generally healthy children.

Regarding cross sectional effects of childhood body composition on bone mineralization, we found that adiposity is likely to have a small specific effect on bone mass beyond its contribution to body size in childhood.

Overall, these findings emphasize the importance of a life course approach to explore the effects of body size and composition on childhood bone mineralization.

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