Accepted Manuscript

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PII: S0261-5614(17)30102-4

DOI: 10.1016/j.clnu.2017.03.011

Reference: YCLNU 3082

To appear in: *Clinical Nutrition*

Received Date: 4 July 2016

Revised Date: 28 January 2017

Accepted Date: 3 March 2017

Please cite this article as: Closa-Monasterolo R, Zaragoza-Jordana M, Ferré N, Luque V, Grote V, Koletzko B, Verduci E, Vecchi F, Escribano J, for the Childhood Obesity Project Group, Adequate calcium intake during long periods improves bone mineral density in healthy children. Data from the Childhood Obesity Project, *Clinical Nutrition* (2017), doi: 10.1016/j.clnu.2017.03.011.

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ABSTRACT

Background: Bone mineralization can be influenced by genetic factors, hormonal status, nutrition, physical activity and body composition. The association of higher calcium (Ca) intake or Ca supplementation with better bone mineral density (BMD) remains controversial. Furthermore, it has been speculated that maintaining long-term adequate Ca intake rather than having a brief supplementation period is more effective. The aim of the study was to prospectively analyse the influence of adequate Ca intake on BMD at 7 years of age in European children. **Methods:** Data from the Childhood Obesity Project were analysed in a prospective longitudinal cohort trial. Dietary intake was recorded using 3-day food records at 4, 5 and 6 years of age. The probability of adequate intake (PA) of Ca was calculated following the American Institute of Medicine guidelines for individual assessments, with FAO, WHO and United Nations University joint expert consultation dietary recommendations. Children were categorised as having high Ca PA (PA>95%) or not (PA<95%). At 7 years, whole body (WB) and lumbar spine (LS) BMD were measured in the Spanish subsample by dual-energy x-ray absorptiometry. Internal BMD z-scores were calculated; BMD below -1 z-score were considered to indicate osteopenia, and BMD z-scores below −2, “low bone mineral density for age”. **Results:** BMD was measured in 179 children. Ca intake at 6 years was positively correlated with LS BMD at 7 years (R=0.205, p=0.030). A Ca increase of 100 mg/d explained 19.4% (p=0.011) of the BMD z-score variation, modifying it by 0.089 (0.021, 0.157) units. Children with Ca PA>95% at 5 and 6 or from 4 to 6 years of age showed higher BMD z-scores at the LS and WB levels than children with Ca PA<95% (p<0.001 and p<0.05 for LS and WB BMD, respectively). Ca PA>95% maintained over 2 years explained 26.3% of the LS BMD z-score variation (p<0.001),

increasing it by 0.669 (0.202, 1.137). PA>95% maintained over 3 years explained 24.9% of the BMD z-score variation, increasing it by 0.773 (0.282, 1.264). The effects of Ca adequacy on WB BMD were similar. Children with PA>95% over 2 years had an Odds ratio of 13.84 and 12 for osteopenia at the LS and WB levels, respectively (p=0.001). **Conclusions:** Long periods of adequate Ca intake in childhood increase BMD and reduce osteopenia risk.

The Childhood Obesity Project clinical trial (CHOP) was registered at clinicaltrials.gov as NCT00338689.

Keywords: Micronutrients - Dietary intake assessment - Intake adequacy assessment – Childhood – Calcium – Bone mineral density.

INTRODUCTION

Maturation of bone occurs during childhood in parallel to growth and development of all body tissues. Calcium (Ca) makes up 19% of body weight and 99% of it is located in the skeleton, in fact its main function is to build and provide rigidity to the bone structure. To ensure normal growth and development of the skeleton and for adequate bone mineralization, it is necessary an adequate intake of Ca during all growth process, especially from birth to 3 years of age when bone mineralization occurs very quickly, during puberty when peak bone mass is reached and until 18-20 years of age when 90-95% of the total body bone mass is achieved. [1–4]. Consequently, a poor bone mineralization is not only a concern for those in adulthood and the elderly, it might be prevented from childhood by guaranteeing adequate conditions to ensure the development of optimal bone mass.

The World Health Organization (WHO) defines normal mineralization as a bone mineral density (BMD) above -1 SD, osteopenia as BMD between -1 and -2.5 SD, and osteoporosis as BMD under -2.5 SD in relation to reference values from a healthy population [5]. As bone mineralization is still occurring during childhood, a BMD under -2 SD is considered low BMD for chronological age [6].

Abbreviations: BMC: Bone Mineral Content, BMD: Bone Mineral Density, BMI: Body Mass Index, Ca: Calcium, DXA: Dual X-Ray Absorptiometry, CHOP: Childhood Obesity Project, FAO: Food and Agriculture Organization of the United Nations, IOM: American Institute of Medicine, LS: Lumbar Spine, PA: Probability of Adequate intake, PAQ-C: Physical Activity Questionnaire for Children, SD: Standard Deviation, WB: Whole Body, WHO: World Health Organization.

Food and Agriculture Organization of the United Nations (FAO), the WHO and the United Nations University joint experts consultation set Ca requirements from 240 to 440 mg/day during the first 9 years of life and on 1018 mg/day from 10 to 18 years [7]. In 2013, a metaanalysis performed with data from several European countries, described Ca mean intakes ranging from 595 to 857, from 563 to 1106 and from 651 to 1487 mg/day in age groups 1-3, 4-10 and 11-18 years, respectively; in the two last age frames, 30 to 57% of subjects had Ca intake under the estimated average requirements [8]. But, although it has been widely studied, the association of higher Ca intake or Ca supplementation with better BMD remains controversial [9–13]. Bone mineralization is a multifactorial issue that is influenced by genetic factors, hormonal status, nutrition (energy, protein, calcium, phosphorus and vitamin D), physical activity and body composition [14,15]. Furthermore, it should be clarified whether the benefits of Ca supply on bone mass are maintained over time. According to some authors, maintaining long-term adequate Ca intake rather than temporary supplementation periods would be more effective [1,13,16].

Thus, our aim was to prospectively analyse whether an adequate Ca supply during long periods in infancy influences bone mineralization.

SUBJECTS AND METHODS

Study design

The present study is a prospective longitudinal cohort trial analysing the influence of Ca intake adequacy during childhood on BMD measured at 7 years of age in Spanish children. This was a secondary study of the Childhood Obesity Project (CHOP), a multicentre, double-blinded, randomized and controlled clinical trial in which 5 European countries (Germany, Belgium, Italy, Poland and Spain) were represented. Breast- and formula-fed infants were enrolled within the first two months after birth, at

a median age of 14 days (IQR: 3–30 days), and were regularly followed until 8 years of age [17].

Study population

All participants from the Spanish subsample of the CHOP who participated in the bone mass mineralization assessment by dual-energy X-ray absorptiometry (DXA) at 7 years old were eligible. All those who took part in the DXA and provided dietary intake information at 4, 5 and/or 6 years of age were included for the present analyses.

Methods

Dietary intake was recorded by parents or caretakers with 3-day weighed/estimated food records (2 week days + 1 weekend day, consecutive) at 4, 5 and 6 years of life, using food scales (Unica 66006; Soehnle, Murrhardt, Germany) and/or a food picture atlas to estimate serving portions (self-designed by the CHOP study nutritionists with demonstrated internal validity, data not published). Intake of energy, protein, calcium, phosphorus and vitamin D were determined (data not shown). Further details on the CHOP study methodology for nutritional assessment have been published elsewhere [18].

The adequacy of Ca intake was quantitatively estimated at the individual level, according to the American Institute of Medicine (IOM) methodology, which is properly described in their guidelines [19]. Due to the multicentre particularities of our study sample, the international estimated average requirements of nutrients developed by the FAO, the WHO and the United Nations University joint experts consultation [7] were the most appropriate for comparisons of our study sample. The probability of adequate Ca intake (PA) was calculated by converting the ratio obtained through the

IOM method into a probability, using the equivalence provided by normal z-score tables and subtracting that value from 100. PA over 95% were considered highly adequate, according to the method, restricting the error to a 5% (equivalent to a statistic 0.05) [19]. Therefore, at each time point, children were categorized as having a high probability of adequate Ca intake if PA>95%, or not, if PA<95%, ensuring that almost all children in PA>95% group had adequate Ca intakes. In order to assess the effect of longitudinal Ca adequate intake on BMD, two longitudinal variables that grouped children with PA>95% during two consecutive years (at 5 and 6 years of age) and during three consecutive years (at 4, 5 and at 6 years) were calculated.

DXA evaluation was performed with the Spanish CHOP study subsample at 7 years of age, using a Lunar Prodigy Primo device, and measured fat mass and bone densitometry. The radiation exposure was 0.4 mGy and 0.9 mGy for the whole body (WB) and for the lumbar spine at L1-L4 (LS), respectively. A 76-KeV X-ray source of energy was used, and the precision error of the test was 1%. The same technician performed all measurements to avoid inter-individual variations. WB and LS bone mineral content (BMC) (g) were measured. Total and lumbar BMD (g/cm^2) were directly obtained from the Lunar device software. BMD was selected to use for comparison in order to adjust bone mineral content by body size. Internal z-scores were calculated for the WB and LS BMD. Children with values of BMD below -1 z-score were classified as children with osteopenia, according to the WHO definitions [7]. Furthermore, those with a BMD z-score below −2 were considered as children with “low bone mineral density for age” [6]. Fat mass index was calculated as fat mass (kg)/ height (m^2). Fat mass index z-scores by gender were calculated using reference values published by Wells et al., 2012 [20].

Physical activity was assessed with the Physical Activity Questionnaire for children (PAQ-C) and was completed by children with the help of their parents or caretakers at 7 years of age. The PAQ-C total score was calculated following its guidelines [21].

The mean of duplicate measures of weight and height at 7 years of age, measured according to the WHO recommendations based on the Lohman reference manual [22], was used. Weight was measured with a Seca 702 scale (Seca, Hamburg, Germany) and was expressed in kg. Standing height was measured with a Seca 242 stadiometer (Seca, Hamburg, Germany) and was expressed in m. Body mass index (BMI) was calculated as weight (Kg)/height $(m)^2$. The z-scores of BMI were calculated using the growth reference values from the United States National Centre for Health Statistics, as recommended by the WHO for children who are 5 to 19 years old [23].

Ethics

The CHOP study protocol and all its amendments were designed following the CONSORT Statement (guidelines for clinical trials) [24] and were in agreement with the Declaration of Helsinki. The study protocol and all its amendments were submitted to and accepted by the Ethics Committees of all the study centres where the study was conducted. Parents or legal representatives of the participating infants received written information and signed an informed consent form before any data were obtained. Additional informed consents were obtained after every new amendment to the original protocol.

Statistical analysis

The frequency of categorical variables was presented as N (%). The distribution of variables was tested with Kolmogorov-Smirnov test and Q-Q graphics of normal

distribution. Pearson correlations were used to determine the linear relationships between Ca intake and BMD. Student's T-tests and Mann-Whitney U tests were used for the statistical cross-sectional comparisons of dietary intake, body composition, anthropometrics and BMD variables between the PA>95% and PA<95% groups, depending on the variable's distribution. Chi squared test was used to compare the frequency of osteopenia according to Ca PA group. Linear regression models were performed to quantify the effect of Ca intake and Ca PA>95% on BMD at 7 years of age, adjusting for anthropometry (BMI at 7 years), physical activity at 7 years and dietary factors as protein, phosphorus and vitamin D intake during childhood (4, 5 and/or 6 years). Collinearity of variables was considered. Binary logistic regressions were performed to determine the risk of osteopenia or low bone mineralization for age at 7 years based on not having a high probability of adequate Ca dietary intake (PA<95%) at different ages. The analyses were adjusted by the same factors as in the linear regression analyses. Statistical significance was accepted at the level $p<0.05$. For the statistical treatment of the data, version 22.0 of the IBM SPSS software was used (IBM Corp., Armonk, NY, USA).

RESULTS

A total of 179 children from the CHOP Spanish subsample participated in the DXA evaluation at 7 years of age, and of those, Ca PA could be estimated for 123, 110 and 112 children at 4, 5 and 6 years of age, respectively.

Calcium intake and bone mineral density

The associations between LS and WB BMD and Ca intake during the years prior to the DXA examination were explored. Significant relationships were only found

between Ca intake at 6 years of age and LS BMD and LS BMD z-score (R=0.205, p=0.030 and R=0.203, p=0.031, respectively).

Ca intake at 6 years had a direct effect on LS BMD z-score (B=0.001, 95% CI 0.000, 0.002), explaining up to 3.3% of its variability at 7 years (p=0.031). After adjusting the model by BMI z-score at 7 years of age, BMI was the variable with the highest effect on BMD, the effect of Ca intake was maintained (B=0.001, CI 95% 0.000, 0.002), and the model explained up to 19.4% of the LS BMD z-score variability (p<0.001). The multivariate model showed that an increase of 100 mg in dietary Ca increased the LS BMD z-score by 0.1.

Other multivariate models adjusting for physical activity and energy, protein, phosphorus or vitamin D intake did not increase the variability in the LS BMD z-score. Neither of the linear regression models assessing Ca intake at earlier ages (5 and 4 years) showed any effect on LS BMD, nor did the models assessing Ca intake at 6, 5 or 4 years of age show any effect on WB BMD z-score (data not shown).

Calcium adequacy according to recommendations and bone mineral density

Children were grouped according to their Ca PA. One group included those with a "high probability of adequate Ca intake" and was called PA>95%, and the other included those with PA<95%. Children with Ca PA>95% at 4, 5, or 6 years tended to have higher LS and WB BMD in comparison to those with Ca PA<95%, although the differences were not significant. The differences became significant when comparing children with a Ca PA>95% that was maintained during consecutive time points (at 5 & 6 or from 4 to 6 years) with those who only had a high probability of adequate intake at some of those time points or at none of those time points (**Figure 1**).

The effects of Ca PA>95% on BMD z-score at both the LS and WB levels were determined through linear regression analyses. The effects on LS and WB BMD z-

scores were stronger the longer Ca PA>95% was maintained over time (**Table 1** and **Table 2**, for LS and WB, respectively).

The dietary factors that could influence BMD, such as energy, protein, vitamin D and phosphorus intake, were significantly higher in the Ca PA>95% group vs. the Ca PA<95% group (data not shown). Therefore, these variables were adjusted for in the regression analyses. No differences in BMI, fat mass index, lean mass index or physical activity were found between groups (data not shown).

Osteopenia and low bone mineral density for age

In our sample, 84.8% of children had normal mineralization, 12.9% had osteopenia, and the remaining 2.3% had low BMD for age at the LS level. At the WB level, the prevalence were 85.4, 12.4 and 2.2%, respectively.

Children with Ca PA>95% at 4, 5, or 6 years of age or during two or three consecutive years (at 5 & 6 or from 4 to 6 years) showed a lower prevalence of osteopenia and low BMD for age compared with those with Ca PA<95% (**Figure 2**).

In the logistic regression analyses, children with Ca PA>95% during 2 consecutive years (5 & 6 years) showed an Odds ratio for osteopenia of 6.8 at the LS level (95% CI 1.2, 37.7; p=0.020) in comparison with those with PA<95%. The Odds ratio was 13.8 when adjusting the models for BMI and dietary factors (95% CI 1.1, 177.0; p=0.001). For the WB, Ca PA>95% showed and Odds ratio for osteopenia of 12.3 when the models were adjusted for BMI and dietary factors (95% CI 1.0, 149.3; p=0.028).

DISCUSSION

To our knowledge, this study is the first to demonstrate in a prospective longitudinal analysis that adequate Ca intake during long periods of time increase later bone mass in healthy children. Our study highlights how having a Ca intake that is over or

under the recommendations during long periods can make a difference. All studies performed previously addressed the amount of Ca consumed, not Ca adequacy. But, as nutrient requirements are different for each individual, the same nutrient intake that is adequate for one individual may be inadequate for another; so in this study, the relationships of both Ca intake and Ca adequacy with BMD at 7 years of age were analysed. These relationships were studied considering Ca adequacy at a single time point and also considering sustained periods of adequacy.

Many studies on the association between BMD and Ca intake have been performed in children. Although it has been widely described that Ca is one of the factors influencing BMC, together with BMI, protein, vitamin D, phosphorus and physical activity, there have still been some controversial results [13–15,25]. According to a review by Rizzoli et al. in 2010 [13], most clinical studies suggest a positive association between Ca intake and bone mass. However, after comparing 13 cross-sectional studies, Lanou et al. [12] concluded that 9 of them did not find a relationship between total dietary Ca intake and BMD or BMC in adolescent girls and women. The remaining 4 studies reported positive associations between dietary Ca and BMD in children aged 6 or older. Lanou et al. [12] also compared 9 prospective studies addressing the influence of total Ca intake at a single time point on later bone mineralization in children and young adults. Eight out of those 9 studies concluded that Ca was not a predictor of BMD, with no significant correlations or associations between Ca intake and BMD being found. Only one prospective study from Lee et al., performed in 1993 with Chinese children from birth to five years, positively associated cumulative Ca intake with later BMD [26]. Even considering the conflicting previously published results, in the Spanish subsample of the CHOP study, we found a positive association of Ca intake at 6 years of age with LS BMD z-score as well as

positive strong effects on LS BMD z-score. The studies in which Lanou et al. [12] found positive associations between Ca intake and BMD were performed in children aged 6 or older, whereas those studies in which no relationship between Ca intake and BMD were found were conducted with an adolescent or adult population. This drives us to speculate that the importance of Ca in relation to bone mineralization is grounded in the period of infancy, while during adolescence, hormonal factors would have a stronger influence on bone mineralization than dietary factors.

But besides analysing Ca intake, as requirements are different for each individual, it also made sense to look for associations between Ca adequacy estimated at the individual level and the BMD of each child. And, in fact, having a Ca PA>95% at 6 years exerted positive effects on LS BMD z-scores, even after adjusting for all the other factors influencing BMC.

Furthermore, when Ca PA was >95% during longer periods (for two or three consecutive years prior to DXA evaluation), the effects on BMD z-score were not only identified at the LS but also at the WB level. In addition, these effects were stronger, consistent with Lee et al., who positively associated long-term cumulative Ca intake with better BMD at a later time [26]. Thus, the effects of Ca on LS and WB BMD were greater when a PA>95% was maintained for long periods of time. Accordingly, we can conclude that Ca absorption into the bone is a continuous and dynamic process, requiring the use of a longitudinal view of Ca intake over time. To detect changes in BMD, Ca intake needs to be adequate for long periods of time, not only at a specific time point. Furthermore, we found slightly different effects of having Ca PA>95% on LS in comparison to WB BMD. While having Ca PA>95% at 6 years only had a significant effect on LS, it was necessary to have a Ca PA>95% for at least two consecutive years to affect WB BMD. This is consistent with the physiological bone

mineralization of the different body areas, as it is well known that LS is the area of the body with the fastest bone turnover, where recent changes in bone mineralization can be observed earlier [27]. Our results were consistent with a review published by Abrams in 2010, which explained how DXA evaluation was used to assess changes in total body Ca. Changes in Ca absorption occurred slowly and needed to be measured over separated months [28]. In addition, in a longitudinal study by Lee et al., cumulative Ca intake from birth to 5 years was positively associated with BMD measured at 5 years; however, current Ca intake at 5 years was not associated with BMD [26].

Regarding the consequences of low BMD, osteoporosis is an important health problem throughout the world. Approximately 33% of women and 20% of men aged 50 or more undergo some type of fracture [29]. As BMD throughout life depends on the peak bone mass reached by the end of the second decade of life, osteoporosis is not only a concern of adulthood and the elderly. To prevent osteoporosis, adequate bone mineralization could be ensured from childhood. During childhood, osteopenia tends to be asymptomatic, thus it is difficult to identify affected patients. The primary sign of osteopenia is the occurrence of fractures after light trauma or during daily activity [30]. In the Spanish subsample of the CHOP study, we performed DXA evaluations with 179 7-year-old healthy children, and we found that 12.8% of children had osteopenia and 2.2% of children had low bone mineral density for age at both the lumbar and whole body levels.

In our sample, having a Ca PA>95% had no effect on the risk of having osteopenia when adequate Ca intake was only maintained at one time point. However, children with Ca PA>95% from 5 to 6 years of age had an Odds ratio of 13 of having

osteopenia at the LS level and an approximately of 12 at the WB level. Therefore, having longer periods of Ca Pa>95% may reduce the risk of osteopenia.

Based on our findings, reaching the dietary recommendations for Ca over long periods of time has an effect on BMD and determines the risk of later osteopenia; thus, we can state that Ca dietary recommendations are completely appropriate, at least for the ages studied herein (from 4 to 6 years).

In conclusion, Ca intake is positively associated with and exerts a positive effect on lumbar spine bone mineral density. The effects of Ca adequacy on both lumbar spine and whole body bone mineral density only occur when Ca is adequately consumed for long periods of time. Furthermore, the same effects occur regarding reductions in osteopenia and/or low bone mineral density for age risk. Therefore, Ca adequacy must be analysed longitudinally to correctly evaluate its effects on bone mineral density.

ACKNOWLEDGEMENTS

We are extremely grateful to the parents and their infants who participated in the Childhood Obesity Project. We are also grateful to the Institut de Diagnòstic per la Imatge of Hospital Universitari Joan XXII de Tarragona, where all DXA evaluations were performed, especially to Francisco Javier Villalba Rubio and Antonio Moreno. We would like to thank the CHOP team for their effort and dedication, especially to the dieticians for their patience in completing and reviewing the nutritional data.

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The presented data are part of a PhD thesis in biomedicine submitted by Marta Zaragoza-Jordana to the medical and health sciences faculty, Universitat Rovira i Virgili, of Reus. The Authors declare no conflicts of interest.

The CHOP trial was funded by the 5th Framework Programme from the European Union. The follow-up of the participants was funded by the 6th Framework Programme (FOOD-CT-2005-007036) and also by the 7th Framework Programme (FP7-KBBE-2007-1, ref. nº 212652; and FP7-289346-EarlyNutrition). This manuscript does not necessarily reflect the views of the Commission and in no way anticipates future policies in this area. No funding bodies had any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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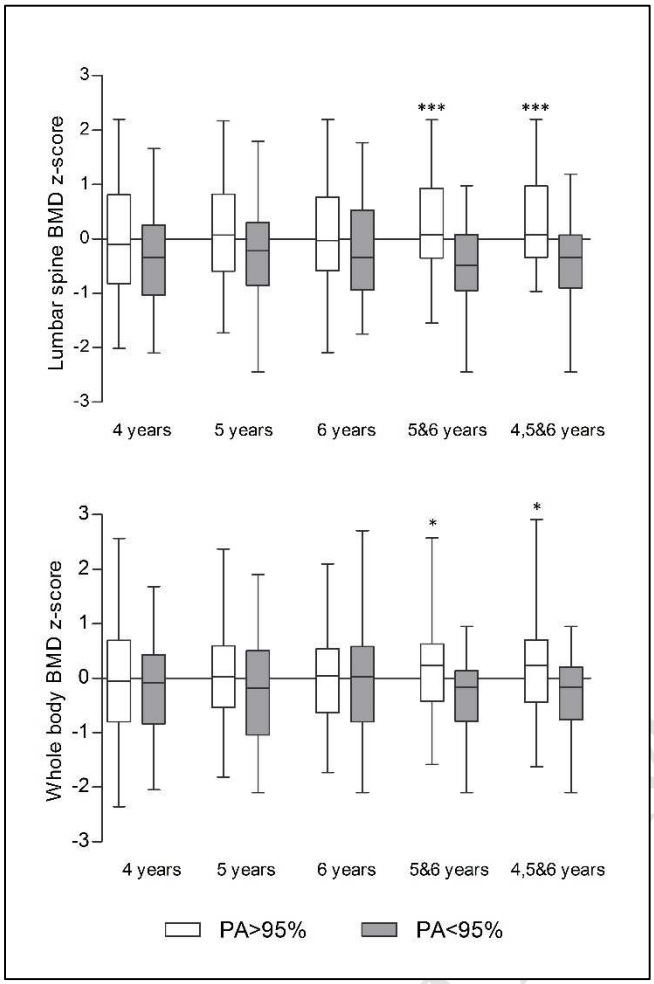
FIGURE 1. Lumbar spine and whole body bone mineral density z-score according to PA group.



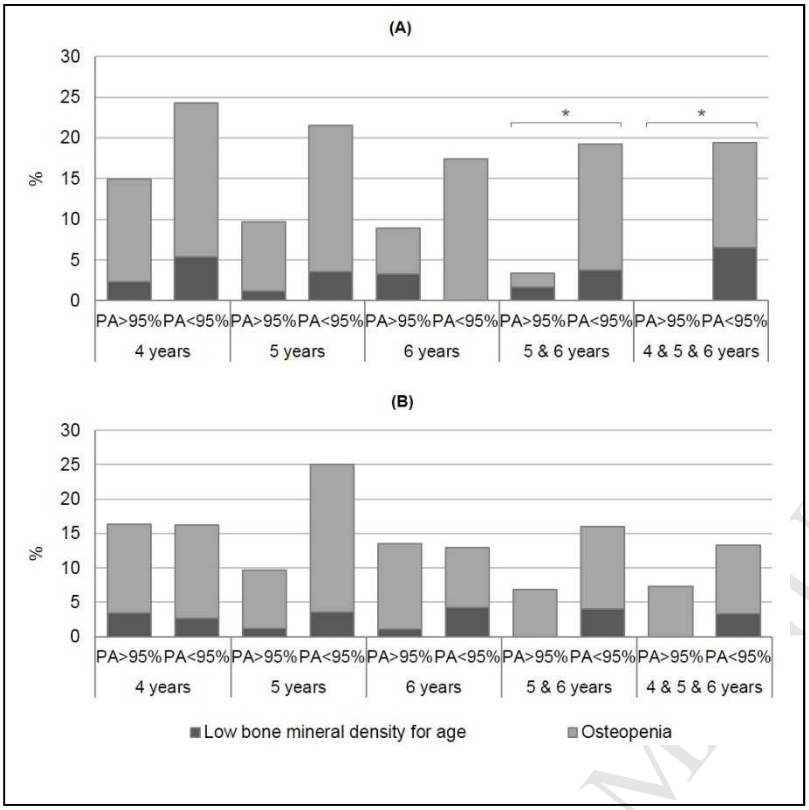
FOOT NOTES: Above, lumbar spine BMD z-score differences according to probability of adequate intake (PA) group. Below, whole body BMD z-score differences according to PA group.

BMD: Bone mineral density. PA>95%= Probability of adequate intake of calcium higher than 95%. PA<95% = Probability of adequate intake of calcium lower than 95%.

*: p<0.05 and ***: p<0.001 vs. children with PA<95%.

FIGURE SIZE: 1 single column (<90mm width)

FIGURE 2. Lumbar spine and whole body osteopenia and low bone mineral density for age according to PA group.



FOOT NOTES: **A:** Differences in lumbar spine osteopenia and low bone mineral density for age according to probability of adequate intake (PA) group. **B:** Differences in whole body osteopenia and low bone mineral density for age according to PA group.

PA>95%= Probability of adequate intake of calcium higher than 95%. PA<95% = Probability of adequate intake of calcium lower than 95%.

*: $p<0.05$ vs. children with PA<95%.

FIGURE SIZE: 1.5 columns with (<140 mm)

TABLE 1. Effects of a high probability of adequate calcium intake on lumbar spine bone mineral density z-score.

	n	β	Confidence interval 95% (min, max)	p-value	R^2	Model p-value
Models with effect on lumbar spine bone mineral density z-score at 7 years of age						
Calcium PA>95% at 6 years [1]	113	0.348	(-0.116, 0.813)	0.140	1.1	ns
Calcium PA>95% at 6 years [2]	113	0.407	(-0.018, 0.833)	0.061	17.2	<0.001
Calcium PA>95% at 6 years [3]	113	0.280	(-0.160, 0.720)	0.210	19.2	<0.001
Calcium PA>95% at 5 & 6 years [1]	85	0.711	(0.297, 1.125)	0.001	11.3	0.001
Calcium PA>95% at 5 & 6 years [2]	85	0.715	(0.331, 1.098)	<0.001	23.9	<0.001
Calcium PA>95% at 5 & 6 years [3]	85	0.607	(0.187, 1.028)	0.005	26.8	<0.001
Calcium PA>95% at 4, 5 & 6 years [1]	72	0.760	(0.340, 1.180)	0.001	14.5	0.001
Calcium PA>95% at 4, 5 & 6 years [2]	72	0.744	(0.342, 1.147)	<0.001	21.6	<0.001
Calcium PA>95% at 4, 5 & 6 years [3]	72	0.764	(0.276, 1.253)	0.003	25.8	<0.001

FOOT NOTES: LS = Lumbar Spine; Calcium PA>95% = High probability of adequate calcium intake; [1]: simple model; [2]: model adjusted by BMI; [3]: model adjusted by BMI and dietary factors; β is the effect of having a high probability of adequate intake on lumbar spine bone mineral density z-score in both the unadjusted ([1]) and adjusted models ([2] and [3]).

TABLE 2. Effects of a high probability of adequate calcium intake on whole body mineral density z-score.

	n	β	Confidence interval 95% (min, max)	p-value	R^2	Model p-value
Models with effect on lumbar spine bone mineral density z-score at 7 years of age						
Calcium PA>95% at 6 years [1]	112	0.062	(-0.384, 0.514)	0.785	-0.8	ns
Calcium PA>95% at 6 years [2]	112	0.110	(-0.315, 0.535)	0.609	11.0	0.001
Calcium PA>95% at 6 years [3]	112	0.028	(-0.409, 0.466)	0.898	11.8	0.001
Calcium PA>95% at 5 & 6 years [1]	84	0.484	(0.077, 0.891)	0.020	5.2	0.020
Calcium PA>95% at 5 & 6 years [2]	84	0.490	(0.100, 0.880)	0.014	13.1	0.001
Calcium PA>95% at 5 & 6 years [3]	84	0.559	(0.089, 1.028)	0.020	17.4	0.001
Calcium PA>95% at 4, 5 & 6 years [1]	71	0.489	(0.082, 0.897)	0.019	6.3	0.019
Calcium PA>95% at 4, 5 & 6 years [2]	71	0.480	(0.079, 0.880)	0.020	9.8	0.011
Calcium PA>95% at 4, 5 & 6 years [3]	71	0.658	(0.187, 1.129)	0.007	20.3	0.002

FOOT NOTES: Calcium PA>95% = High probability of adequate calcium intake; [1]: simple model; [2]: model adjusted by BMI; [3]: model adjusted by BMI and dietary factors; β is the effect of having a high probability on adequate intake on whole body bone mineral density z-score in both the unadjusted ([1]) and adjusted models ([2] and [3]).