Bone turnover in pregnancy, measured by urinary C-terminal telopeptide of type I collagen (CTX), is influenced by vitamin D supplementation and is associated with maternal bone health: Findings from the MAVIDOS trial

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Short running head: Vitamin D supplementation in pregnancy and bone resorption

Abbreviations list (if there are three or more):

25(OH)-vitamin D, 25(OH)D 25-hydroxycholecalciferol, calcidiol

1,25(OH)$_2$D 1,25-diydroxycholecalciferol, calcitriol

24,25(OH)$_2$D 24,25- diydroxycholecalciferol

CLIA Chemiluminescent immunoassay

CTX C-terminal telopeptide of type I collagen

DXA Dual energy X-ray absorptiometry

ELISA Enzyme Linked Immunosorbent Assay

FGF-23 Fibroblast growth factor-23

MAVIDOS The Maternal Vitamin D Osteoporosis Study

MHRA United Kingdom (UK) Medicines & Healthcare products Regulatory Agency

NTX N-terminal telopeptide of type 1 collagen

OPG Osteoprotegerin

P1NP N-terminal collagen extension propeptide

PTH Parathyroid Hormone

PTHrp Parathyroid hormone- related peptide

RANKL Receptor activator of NF-κB ligand

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Data sharing: Data described in the manuscript (de-identified), code book, and analytic code will be made available upon request pending application to and approval of the corresponding author.

Abstract

Background:

The pattern of change in maternal bone turnover throughout pregnancy is poorly characterized.

Objective:

We investigated changes across pregnancy in a marker of maternal bone resorption, urinary C-terminal telopeptide of type I collagen (CTX), the influence of gestational vitamin D supplementation, and associations between CTX and maternal postnatal bone indices.

Design:

The Maternal Vitamin D Osteoporosis Study (MAVIDOS) is a randomized, double-blind, placebo-controlled trial of 1000 IU/day cholecalciferol vs placebo from 14 weeks' gestation to birth. Maternal second void urinary α- and β-CTX were measured (Enzyme Linked Immunosorbent Assay, ELISA) at 14 and 34 weeks' gestation; DXA was performed within 2 weeks post-partum. Mann-Whitney rank sum, Spearman's rank correlation and linear regression were used to compare median CTX values within and between groups from early to late pregnancy, and associations with maternal bone outcomes.

Results:

372 women had CTX and 25(OH)-vitamin D measured in early and late pregnancy. CTX at 14 and 34 weeks' gestation were correlated in both placebo (r=0.31) and cholecalciferol (r=0.45) groups (p<0.0001). Median CTX(μg/mmol creatinine) increased from 14 to 34 weeks' gestation in both groups (n=372 total) [placebo (n=188) 223.6 to 449.7; cholecalciferol (n=184) 222.3 to 419.3; p=0.03 for placebo vs cholecalciferol difference in CTX at 34 weeks' gestation]. The conditional increase in CTX(SD) from early to late pregnancy was greater in the placebo group (n=188) than in the cholecalciferol group (n=184) [placebo, mean(SD) 0.16(0.92); cholecalciferol, -0.16(1.06); p difference<0.01]. Higher CTX at 34 weeks' gestation was associated, similarly in both groups, with

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lower maternal total hip and lumbar spine bone mineral content (BMC) and bone mineral density, e.g. lumbar spine BMD [β= -0.02g/cm^2/SD increase in CTX; 95%CI: -0.027, -0.002; p=0.02, n=283].

Conclusions:

Maternal urinary CTX, a bone resorption marker, rises through pregnancy, though to a lesser degree with gestational cholecalciferol supplementation, and is inversely associated with maternal bone mass post-partum.

Key words: osteoporosis; epidemiology; vitamin D; bone turnover; pregnancy; CTX; DXA

Introduction

The human fetal skeleton begins to mineralise between the 8 and 12 weeks' gestation when primary ossification centers form in the vertebrae and long bones (1). Approximately 30g of calcium are required in total for its development, 80% of which is accrued in the 3rd trimester. Maternal calcium homeostasis adapts to meet the calcium demands of the developing fetus (1-3), particularly through increased intestinal absorption and renal reabsorption of calcium. The maternal skeleton's role in supplying the fetus, and how it might respond to interventions such as vitamin D supplementation, are not well defined, because of the difficulty in using dual energy X-ray absorptiometry (DXA), which involves ionising radiation, in pregnancy. Biochemical markers of bone turnover offer a non-invasive method of monitoring changes in bone resorption or formation during pregnancy (4), and provide some insight into the impact of pregnancy on maternal bone and its contribution to fetal development. There are two groups of bone turnover markers: markers of bone formation (such as N-terminal collagen extension propeptide (P1NP), bone alkaline phosphatase) and markers of resorption (such as C-terminal cross-linking telopeptide of type 1 collagen (CTX), N-terminal telopeptide of type 1 collagen (NTX), deoxypyridinoline, hydroxyproline). In the vitamin D deficient state, plasma calcium concentrations may be reduced with a consequent rise in parathyroid hormone, leading to increased bone resorption (5). Indeed at the population level, there is evidence of inverse associations between

25(OH)-vitamin D concentrations and markers of bone resorption such as CTX (6-9). However, few studies have investigated these relationships across normal pregnancies, or the influence of vitamin D supplementation (10-17).

The MAVIDOS trial of vitamin D supplementation in pregnancy (of which the primary aim was to investigate the effect of gestational cholecalciferol supplementation on offspring bone mass) provides an opportunity to study the impact of this supplement on a marker of maternal bone resorption (CTX) and maternal post-partum bone indices (18, 19). As the basis for this post hoc analysis, we hypothesised that vitamin D supplementation in pregnancy would result in reduced osteoclastic bone resorption, and thus that we would observe: a) an inverse association between vitamin D supplementation (or 25(OH)-vitamin D concentration) and CTX concentrations, and b) an inverse association between maternal CTX concentrations and measures of maternal post-partum bone mass.

Subjects and Methods

The Maternal Vitamin D Osteoporosis Study (MAVIDOS)

MAVIDOS was a multicenter, double-blind, randomized, placebo-controlled trial of vitamin D supplementation in pregnancy. The primary outcome was neonatal bone mass. A detailed description of the study methods (18) and primary findings have been published previously (19).

Briefly, women attending one of three UK hospitals [University Hospital Southampton NHS Foundation Trust, Southampton, UK; Oxford University Hospitals NHS Foundation Trust, Oxford, UK; Sheffield Teaching Hospitals NHS Foundation Trust (University of Sheffield), Sheffield, UK] for early pregnancy ultrasound screening (11-14 weeks' gestation) between 6th October 2008 and 11th February 2014 were invited to participate in the study (19, 20). Inclusion criteria were: age over 18 years, singleton pregnancy, and gestation less than 17 weeks based on last menstrual period and ultrasound measurements. Women with known metabolic bone disease, renal stones, hyperparathyroidism or hypercalciuria; those taking medication known to interfere with fetal growth, those with fetal anomalies on ultrasonography and women already using >400IU/day vitamin D

supplementation were excluded. A screening blood sample was obtained and analysed on the local NHS platform [all three laboratories (Southampton, Oxford and Sheffield) participate in DEQAS vitamin D quality assurance system (http://www.deqas.org/)]. Women with 25(OH)D between 25 and 100nmol/l and serum calcium <2.75mmol/l were eligible to enrol fully in the study. Participants were randomized in a double-blind design to either cholecalciferol 1000 IU/day or matched placebo [Merck KGaA, (Darmstadt, Germany)/ Sharp Clinical Services (Crickhowell, UK; previously DHP-Bilcare)], which was commenced before 17 weeks' gestation. All participants received standard antenatal care and could continue self-administration of dietary supplements containing up to 400 IU/day vitamin D. Data from Southampton participants were used in the current analysis as CTX was not measured in Oxford and Sheffield participants.

Maternal assessments during pregnancy

Prior to commencing the study medication, and again at 34 weeks' gestation, the women attended the hospital for a detailed assessment of diet (including supplement use), lifestyle (smoking, physical activity, employment) and health (past medical history, current medication use) using interviewer-led questionnaires. Ethnic group was self-reported by the participant as "White, Black Caribbean, Black African, Black Other, Indian, Pakistani, Bangladeshi, Chinese, Other Asian group or Other (specify)". This was then categorized as White or non-White. Anthropometric measurements included height, measured to the nearest 0.1cm using a stadiometer (Seca, Birmingham, UK), and weight, assessed to the nearest 0.1kg using calibrated electronic scales (Seca, Birmingham, UK).

Assessment of 25(OH)-vitamin D and CTX

On the day that the study medication was dispensed and at 34 weeks' gestation, a non-fasted venous blood sample was obtained, and serum stored at -80°C. 25(OH)-vitamin D [25(OH)D] was assessed by chemiluminescent immunoassay (CLIA) (Liaison automated platform, Diasorin, Minnesota, USA). All samples were analysed in a single batch at the end of the study at MRC Human Nutrition Research, Cambridge, UK. Details of assay performance and quality control through participation in DEQAS, NIST and NEQAS are given elsewhere (21, 22).

Participants were asked to provide a second-void early morning urine sample to measure urine α and β-C-terminal telopeptide of type I collagen (CTX) on the day the study medication was started (around 14 weeks' gestation), and again at 34 weeks' gestation. Owing to the diurnal variation in CTX, in addition to the practical difficulties of obtaining a fasted blood sample from pregnant women, second-void early morning urine collection by the participant at home was selected as the most appropriate measure of bone resorption. All samples were stored at -70°C and those from Southampton participants were sent in a batch for measurement by ELISA (Immunodiagnostic Systems, Boldon, UK) at the Academic Unit of Bone Metabolism, University of Sheffield, Sheffield, UK. Total CTX (sum of α and β forms) is expressed as a ratio to urine creatinine (μg/mmol) (23). The assay had an inter-assay coefficient of variation of 6% (24).

Maternal and neonatal DXA

Mothers and neonates underwent DXA assessment ; mothers underwent DXA at the lumbar spine and hip sites, neonates at the whole body and lumbar spine sites (instrument at the Southampton center: Hologic Discovery, Hologic Inc, Bedford, MA, USA) within 2 weeks of delivery. The instrument underwent daily quality control. To maximise the scan quality, infants were undressed, clothed in a standard towel, fed, and pacified before the assessment. The total radiation dose was estimated to be 0.04 mSv, equivalent to about 7 days' exposure to background radiation in the UK. All DXA images were reviewed for movement artefacts and quality by two operators who were blinded to treatment allocation.

Ethics

The trial was approved by the Southampton and South West Hampshire Research Ethics Committee (07/H0502/113). MAVIDOS was registered prospectively (ISRCTN:82927713; EUDRACT:2007-001716-23); full approval from the United Kingdom (UK) Medicines & Healthcare products Regulatory Agency (MHRA) was granted, and written, informed consent was obtained from all participants.

Statistics

Women who had urinary CTX and 25(OH)D measured at either 14 and 34 weeks' gestation, had not taken cholecalciferol supplements of greater than 400 IU daily in addition to the study intervention and who delivered a liveborn infant, were included in the analysis. All outcomes were assessed for normality using visual inspection. Maternal characteristics in the placebo and vitamin D supplemented groups were documented. Comparisons between median CTX concentrations in the placebo and cholecalciferol supplemented groups in early pregnancy (around 14 weeks' gestation) and late pregnancy (34 weeks' gestation) were made using the Mann-Whitney Rank Sum test. In order to avoid problems from regression to the mean and correlation between baseline value and change, early and late pregnancy CTX were summarised into a change in CTX variable, calculated as the residual from the regression of the later value on the earlier, differences between groups were tested using a t-test (25). This change in CTX variable was then used as an outcome variable in linear regression to explore maternal factors associated with change. Change in CTX was also used as a predictor in regression analyses examining associations between maternal CTX and neonatal bone indices.Spearman's correlation was used to test associations between CTX in early pregnancy and late pregnancy, and between CTX (in early or late pregnancy) and maternal serum 25(OH)D measurements (in early or late pregnancy).

The distribution of CTX in early and late pregnancy was positively skewed and was therefore transformed and standardised using a Fisher-Yates Transformation for inclusion in regression analyses. Fisher-Yates transformed CTX values are used as a predictor in regression analysis with DXA-assessed bone outcomes ; beta coefficients represent change in bone outcome per 1 SD increase in CTX. In order to remove the additional influence of season on 25(OH)D concentrations, further analyses included adjustment for season of birth, using UK Meteorological Office classifications were used; Winter (December-Feb), Spring (March-May), Summer (June-August), Autumn (September-November). Covariates identified as associated with maternal CTX concentrations and known to be

associated with bone mass were included in CTX-bone regression models. All analyses were performed in Stata v14 (Statacorp, College Station, Texas, USA).

Results

Characteristics of the participants

965 women recruited to the MAVIDOS trial remained in the study until delivery, and of these 630 had CTX measured in either early (n = 493) or late pregnancy (n = 498). 372 women had a CTX measurement at both timepoints (placebo group, n = 188, cholecalciferol supplemented group, n = 184), as shown in **Supplementary Figure 1**. The characteristics of the mothers with a measurement of urine CTX in early or late pregnancy are summarised in **Table 1**, demonstrating a mean age of 30.4 years, 95.0% of self-reported White ethnicity and 44.5% nulliparous. There was no evidence of selective drop-out (as shown in **Supplementary Table 1**, comparing the participants of this sub-study with the overall MAVIDOS trial) and thus in this subgroup analysis baseline characteristics appeared comparable between the mothers randomized to placebo or cholecalciferol. Baseline maternal 25(OH)D concentration (measured at recruitment, 14 weeks' gestation) was similar in both groups. At 34 weeks' gestation mean maternal 25(OH)D concentration was significantly higher in the cholecalciferol supplemented group [66.0 nmol/l (SD 20.4)] than in those who received placebo [42.5 nmol/l (SD 20.6); p < 0.0001].

Maternal urinary CTX in early and late pregnancy

As demonstrated graphically in **Figure 1**, both early and late pregnancy urinary CTX were in a positively skewed distribution: a few women had very high CTX measures (> 1500 μg/mmol creatinine). In both the placebo and 1000 IU/day cholecalciferol supplemented mothers, urinary CTX increased markedly from 14 to 34 weeks' gestation [median 14 and 34 week CTX: placebo group, 223.8 and 445.3 μg/mmol creatinine; cholecalciferol supplemented group, 217.5 and 420.0 μg/mmol creatinine; (both p-difference < 0.0001)], as shown in **Table 2**. Median CTX at 34 weeks' gestation tended to be greater in the placebo group compared with the cholecalciferol supplemented group

(mean difference 25.3 µg/mmol; p=0.06). Restricting the analysis to women with CTX measures in early and late pregnancy (n=372) led to greater observed differences in median CTX at 34 weeks' gestation (30.4 µg/mmol greater in the placebo group than the cholecalciferol supplemented group (p=0.03), also shown in **Table 2**. The conditional increase in CTX(SD) from early to late pregnancy was greater in the placebo group than in the cholecalciferol group [placebo, mean(SD) 0.16(0.92); cholecalciferol, -0.16(1.06); p difference<0.01]. Note that the negative conditional change in CTX in the cholecalciferol group is relative to the overall distribution of standardised conditional change in CTX so still represents a rise: both groups demonstrated a large, positive absolute increase in CTX (Placebo, median percentage change from early to late pregnancy was 111.1% (IQR 47.1, 210.9); cholecalciferol supplemented, 88.8% (IQR 25.9, 183.1)). When stratified by baseline 25(OH)-vitamin D status in early pregnancy [sufficient, ≥50nmol/l; or insufficient, below 50nmol/l], classified according to the 2011 report on dietary requirements for calcium and vitamin D from the Institute of Medicine (IOM)(2)), a threshold effect was observed. Women with vitamin D insufficiency at baseline in the placebo group had a greater conditional increase in CTX (0.28 SD, n=126) than in the cholecalciferol supplemented group (-0.15SD, n=123), p difference <0.01). In women with vitamin D sufficiency, similar conditional increases were observed in the placebo and cholecalciferol supplemented groups, as shown in **Table 2.**

Maternal factors associated with CTX in early and late pregnancy

Table 3 documents the univariate associations between maternal characteristics and CTX concentrations in early or late pregnancy and with conditional change in CTX. Greater maternal age, parity and height were associated with lower CTX in early and late pregnancy. Cholecalciferol supplementation (1000 IU/day) from 14 weeks' gestation was associated with -0.18 SD lower late pregnancy CTX compared with those who received placebo [95% CI -0.35, -0.001 (p = 0.05)]. There was evidence of associations between lower conditional change in CTX from early to late pregnancy and: lower BMI, greater baseline 25(OH)D, and cholecalciferol supplementation (-0.32SD conditional change in CTX compared to those who received placebo [95% CI -0.52, -0.11, p=0.002]).

Associations between early and late pregnancy urinary CTX and maternal serum 25(OH)D

There were modest correlations between early and late pregnancy CTX in both the placebo and treatment groups (placebo group r=0.31, treatment group r=0.45, both p<0.0001), **Supplementary Table 2**. Indeed, both early and late pregnancy 25(OH)D were also correlated, with evidence of a greater magnitude of correlation in the placebo group (r=0.36, p<0.0001) than in the cholecalciferol supplemented group (r=0.22, p=0.0003). No major correlations were demonstrated between early or late pregnancy serum 25(OH)D measurements and CTX measures. In the UK, season is strongly associated with maternal 25(OH)D in pregnancy.(19) CTX concentrations by season are presented in **Supplementary Table 3**, demonstrating little evidence for any seasonal effect.

In multivariate regression analyses adjusting for season of delivery (**Table 4**) we observed a trend towards modest inverse associations between late pregnancy maternal 25(OH)D and CTX in late pregnancy [in both groups combined, β = -0.004 SD/nmol/L greater 25(OH)D (95%CI-0.008, -0.001), p=0.056 and also with conditional change in CTX [β = −0.008 (−0.012, −0.003) SD/ nmol/l greater 25(OH)D, p=0.001. Associations between early pregnancy 25(OH)D and conditional change in CTX were observed, but were less robust (p=0.049). Adjustment for maternal age, smoking, parity, BMI and height (factors which were associated with CTX measures) did not materially alter the observed trends.

Maternal CTX in late pregnancy, conditional change in CTX, and maternal postpartum bone heath

In the 283 mothers with a late pregnancy measure of urine CTX and postpartum DXA, greater maternal urinary CTX at 34 weeks' gestation was associated with lower maternal post-partum lumbar spine and total hip bone mineral content (BMC) and areal bone mineral density (aBMD), following adjustment for maternal age, parity, smoking and BMI in early pregnancy (in the groups combined). These associations appeared more robust in the cholecalciferol than in the placebo group and CTX-bone associations are summarised in **Table 5** and **Figure 2** (groups combined). The direction of associations with conditional change in CTX (**Table 5**) followed a similar but non-significant pattern and were substantially attenuated compared with those for LP CTX. Additional adjustment for maternal height attenuated the observed relationships (**Supplementary Tables 4a and b**).

Conditional change in CTX, and neonatal bone indices

321 mother – neonate pairs had a measure of early or late pregnancy maternal CTX and a neonatal whole body or lumbar spine DXA. The characteristics of the neonates are presented in **Supplementary Table 5**. No differences in neonatal anthropometry or DXA characteristics were observed between the placebo and cholecalciferol supplemented groups. On regression analyses (**Table 6**) greater maternal conditional change in CTX was associated with greater neonatal total body and total spine area, BMC and aBMD (adjusted for neonatal sex, age at DXA scan and gestational age at delivery). The magnitude and direction of associations was similar between the placebo and cholecalciferol supplemented groups, no interaction was observed between conditional change in CTX and randomisation group.

Discussion

In this post-hoc analysis of the MAVIDOS randomized controlled trial (RCT), we have demonstrated that maternal gestational cholecalciferol supplementation is associated with a smaller gestational increase in bone resorption markers compared with placebo. Whilst maternal urinary CTX almost doubled from 14 weeks' to 34 weeks' gestation in both vitamin D supplemented and placebo groups, the conditional increase in CTX from early to late pregnancy was lower in the cholecalciferol supplemented compared with the placebo group, with greater differences in conditional increase in CTX between the groups observed in vitamin D insufficient women. Furthermore, late pregnancy CTX was inversely associated with post-partum measures of maternal bone from DXA.

Our observation of increasing markers of bone resorption throughout pregnancy is replicated in other smaller studies, in which bone resorption markers have been shown to rise markedly through gestation, whilst markers of bone formation tend to be low in early pregnancy, reaching normal values by term, such that there is a net resorptive state in late pregnancy (10-17, 27). Studies of maternal bone mass throughout pregnancy and the post-partum period support this, showing that BMD declines by a small amount (between 2% and 5% at both trabecular (28) and cortical sites in the axial and peripheral skeleton (29), but importantly, pregnancy does not have a negative impact on bone health in later life (30). Both circadian (peaking in the early morning) and seasonal (peaking during winter months) variations in bone resorption markers have been observed (7, 31). Vitamin D has been

proposed to play a role in this seasonal variation. Again, in keeping with our findings, several studies have demonstrated a negative association between 25(OH)D status and markers of bone resorption, in both non-pregnant and pregnant women (6-9). Our observation that greater conditional increases in CTX were associated with greater neonatal bone mass is in keeping with a previous study from our group using the Southampton Women's Survey cohort. Greater maternal bone loss in pregnancy (as measured by calcaneal ultrasound) was shown to be associated with increased bone mineral density in their babies (32), consistent with associations between maternal bone resorption and transplacental calcium transfer to the developing fetus.

The link, however, between maternal vitamin D and its modulation of calcium transfer to the fetus is less clear. Circulating $1,25(OH)_2D$ (calcitriol) increases during pregnancy with (most importantly) the maternal kidney and also possibly the placenta providing the necessary $1-\alpha$-hydroxylase activity (11, 16, 33). Increased $1,25(OH)_2D$ is thought to contribute to greater maternal intestinal calcium absorption (34). Several factors are known to contribute to the regulation of the balance between maternal and fetal bone formation and resorption (osteoblast and osteoclast activity), including parathyroid hormone (PTH) and parathyroid hormone- related peptide (PTHrp) (35-37), receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG), Sclerostin and fibroblast growth factor-23 (FGF-23), the Wnt pathway, oestrogens and prolactin (38). It is, therefore, unlikely that the study of one potential mediating factor (maternal serum 25(OH)D concentrations), and one measure of bone resorption (urinary CTX), is likely to provide conclusive information about the system, though it does provide an important step towards our understanding of this complex process. Indeed, the European Food Safety Authority has identified the associations between vitamin D, bone turnover markers and bone health as a current knowledge gap, as outlined in their 2016 scientific opinion paper (39).

To our knowledge, MAVIDOS represents the first opportunity to study vitamin D and pregnancy bone markers in an interventional setting. The mechanism for the observed inverse relationship between maternal cholecalciferol supplementation vs placebo and CTX concentrations could be related to greater calcium availability from maternal intestinal absorption for the developing fetus in

mothers with improved 25(OH)D status, reducing the requirement for the release of calcium by maternal bone resorption. Our findings that higher maternal serum 25(OH)D concentration in late pregnancy is associated with lower late pregnancy CTX, after adjustment for season, and that women with vitamin D insufficiency in early pregnancy had greater conditional increases in CTX throughout pregnancy, supports this hypothesis. Studies of calcium and vitamin D in pregnant women provide supportive evidence for the resulting notion that maternal 25(OH)D status in late pregnancy is important in regulating maternal bone turnover to ensure calcium availability for the developing fetus. One previous RCT of calcium supplementation in pregnancy showed that daily consumption of a 1100 mg calcium supplement led to a 15% reduction in urinary NTX during the second and third trimesters (40). A prospective cohort showed that higher calcium intake in late pregnancy was associated with reduced bone resorption, with greater effects in winter, when vitamin D mediated calcium absorption is less (9). Other longitudinal studies of maternal pregnancy 25(OH)D status demonstrated that vitamin D repletion was associated with lower markers of bone resorption. In two small studies (n=47 and n=60) of pregnant women and healthy non-pregnant controls, greater 25(OH)D status, and greater levels of the vitamin D metabolites [1,25(OH)$_2$D, 24,25(OH)$_2$D] tended to be associated with lower bone resorption markers (serum NTX and CTX respectively) in the second and third trimesters and post-partum period, whilst no correlation between 25(OH)D and bone resorption markers was seen in the non-pregnant controls (6, 8). In non-pregnant women, a high bone turnover in those who are vitamin D deficient has been observed, with vitamin D supplementation attenuating this effect in some studies (41) but not others (42, 43).

CTX, released by osteoclastic resorption of bone, is significantly correlated with lower total hip BMD, distal radius BMD and lumbar spine BMD and poorer bone indices assessed by pQCT and histology (44-46). Our finding that greater urinary CTX in late pregnancy was associated with lower lumbar spine and hip BMC and BMD is in keeping with this observation. Interestingly the associations were of consistent direction but attenuated with conditional change in CTX which may reflect lower statistical power given the smaller cohort with measures at both timepoints or potentially that the absolute value in late pregnancy rather than change in concentrations is the more important

factor. Notably, although the magnitude of the associations between CTX and bone measures were attenuated after adjustment for maternal height, this was a modest effect on aBMD, suggesting CTX-aBMD relationships at least partly independent of linear skeletal size.

Limitations

Our study had some limitations, the first being that, as a result of ethical stipulations, participants with 25(OH)D concentrations less than 25mmol/L at screening at 12 weeks of gestation could not be included. However, vitamin D deficiency has developed in a proportion of women by gestational week 34, particularly in the placebo group and those that with winter deliveries. Our findings may also not be relevant to non-White populations, as our study did not include many women who were not of self-reported White ethnicity. Additionally, we could not exclude the possibility that some participants were taking vitamin D in addition to the study drug, though reported supplement use did not differ between groups at baseline interview. Furthermore, as a result of the primary trial design, we were unable to characterize dietary calcium intake in detail, and assays of vitamin D metabolites $(1,25(OH)_2$ vitamin D, $24,25(OH)_2$ vitamin D), PTH and PTHrp were not available, meaning a comprehensive assessment of maternal factors determining bone turnover could not be made. Limitations regarding the use of CTX as a marker of bone resorption should also be recognised including its circadian rhythm and relationship with food intake (though early morning, second void urine was used to minimise this variation). We were not able to adjust fully for hemodilution in pregnancy (which may also impact upon serum 25(OH)D levels)(47) and individual changes in maternal glomerular filtration rate, but did use the CTX value relative to creatinine to account for renal function (13). The assay used in this study does not distinguish between the different isoforms of CTX.α−CTX in particular is predominantly released from growing fetal bone and may cross the placenta. One study estimated that approximately 9% α−CTX and 2% of β−CTX in the mother's urine in late pregnancy may be coming from the fetal skeleton(48); therefore, it is not possible to quantify the fetal contribution to the mother's urinary CTX. As the participants were recruited in early pregnancy, pre-pregnancy DXA scans could not be performed. Therefore, we did not have the opportunity to explore changes in BMC and BMD, and its relationship with CTX, from pre-pregnancy

to postpartum. Maternal breastfeeding is known to play a large role in determining levels of bone resorption and postpartum BMD, particularly at trabecular sites (49); lumbar spine BMD has been shown to decline by a mean of 5-10% during exclusive breastfeeding (49). Though mothers underwent DXA within 2 weeks postpartum, the number of days spent breastfeeding prior to DXA assessment may be important but these data were not available. Finally, it should be acknowledged that this is a sub-analysis of an RCT, so whilst the differences in CTX between groups and associations with bone indices are biologically plausible and consistent with existing medical literature, they should be recognised as post hoc and require replication.

In conclusion, we have shown that maternal urinary CTX rises from early to late pregnancy, with the magnitude of this rise appearing to be reduced by gestational cholecalciferol supplementation, and is inversely associated with maternal bone mass post-partum. These findings are consistent with a protective effect of gestational vitamin D supplementation on maternal bone health and inform our understanding of bone resorption in pregnancy and the potential relationship with vitamin D metabolism. Long-term follow-up of both mothers and offspring, with repeat assessments of bone indices, will enable us to determine better the lasting impact of cholecalciferol supplementation in pregnancy.

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Author contributions

EMC and NCH designed the research, EMC, KM, RJM and NCH wrote the paper. FG and RE, alongside IS and AP undertook or advised upon the laboratory analyses. CP, SD, SRC and HMI performed the statistical analysis. NJB, SHK, ATP, RF, SVG, KMG, MKJ oversaw the MAVIDOS trial at the Oxford, Sheffield and Southampton sites. NCH and CC supervised the study and the preparation of the manuscript. CC had primary responsibility for final content. All authors contributed to the preparation and have read and approved the final version.

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Table 1: Characteristics of MAVIDOS mothers with a measurement of CTX available in early or late pregnancy, in the study overall, and in the placebo group and cholecalciferol supplemented group separately, n = total number of participants with available data, N = number with characteristic.

Baseline characteristic	All			Placebo			Cholecalciferol 1000 IU/day		
	n	Mean	SD	n	Mean	SD	n	Mean	SD
Age [1]	607	30.4	5.2	296	30.4	5.3	311	30.3	5.3
Height (cm) [1]	602	165.4	6.5	292	165.5	6.7	310	165.2	6.4
Weight (kg) [1]	607	71.8	15.0	296	72.8	14.4	311	70.8	15.5
Pregnancy weight gain (kg)[2]	536	9.5	3.6	264	9.3	3.7	272	9.7	3.4
25(OH)D at 14 weeks (nmol/l)	630	44.6	16.2	312	44.1	16.1	318	45.0	16.3
25(OH)D at 34 weeks (nmol/l)	561	54.2	23.6	282	42.5	20.6	279	66.0	20.4
Adjusted calcium at 14 weeks (mmol/l)	630	2.38	0.08	312	2.38	0.09	318	2.37	0.07
Adjusted calcium at 34 weeks (mmol/l)	560	2.34	0.11	280	2.34	0.12	280	2.34	0.10
	n	Median	IQR	n	Median	IQR	n	Median	IQR
BMI (kg/m^2) [1]	602	24.9	22.5-28.6	292	25.5	22.9-29.5	310	24.4	22.3-28.1
	n	N	%	n	N	%	n	N	%
First Pregnancy	604	269	44.5	294	134	45.6	310	135	43.5
White (Caucasian) ethnicity	604	574	95.0	296	285	96.3	308	289	93.8
A Level or higher qualification	602	450	74.8	293	214	73.0	309	236	76.4
Smoker [1]	605	54	8.9	295	27	9.2	310	27	8.7
More than one hour strenuous exercise per week [1]	553	79	14.3	270	33	12.2	283	46	16.3

[1] Characteristics at early pregnancy study visit; [2] pregnancy weight gain = 34 week maternal weight – 14 week maternal weight

Table 2: Maternal urinary CTX in early (EP, 14 weeks) and late pregnancy (LP, 34 weeks) and conditional change in CTX from early to late pregnancy, in all mothers in the MAVIDOS trial CTX sub-study with a measure of early or late pregnancy CTX, and in the placebo group and cholecalciferol 1000 IU supplemented groups separately; all mothers with a measure of both early and late pregnancy CTX, and in the placebo group and cholecalciferol 1000 IU supplemented groups separately. Change in CTX is calculated with a regression model, 34 week CTX as dependent variable and adjusting for 14 week CTX, absolute change in CTX is also shown. Participants are further stratified according to vitamin D sufficiency (≥50nmol/l or insufficiency <50nmol/l) in early pregnancy.

	All			**Placebo**			**Cholecalciferol 1000 IU/day**			**p-value**
	n	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**	**Median**	**IQR**	
Participants with EP or LP CTX measures										
EP CTX (µg/mmol creatinine)	493	219.4	154.7, 306.4	242	223.8	155.0, 308.6	251	217.5	154.5, 305.2	N/A
LP CTX (µg/mmol creatinine)	498	437.4	292.3, 633.0	252	445.3	317.6, 657.3	246	420.0	252.2, 608.5	0.06
Participants with both EP and LP CTX measures										
EP CTX (µg/mmol creatinine)	372	223.0	159.5, 316.4	188	223.6	153.6, 311.8	184	222.3	169.2, 319.8	N/A
LP CTX (µg/mmol creatinine)	372	429.6	291.0, 660.6	188	449.7	327.3, 687.2	184	419.3	238.9, 629.9	0.03
	n	**Mean**	**SD**	**n**	**Mean**	**SD**	**n**	**Mean**	**SD**	**p-value**
Δ CTX (z score)				188	0.16	0.92	184	-0.16	1.06	<0.01
Δ CTX (absolute)				188	258.1	322.1	184	214.5	316.7	0.19
Vitamin D sufficient women, baseline 25(OH)D ≥50nmol/l										
	All			**Placebo**			**Cholecalciferol 1000 IU/day**			**p-value**
	n	**Median**	**IQR**	**n**	**Median**	**IQR**	**n**	**Median**	**IQR**	
EP CTX (µg/mmol creatinine)	168	232.8	167.0, 317.6	81	239.3	175.2, 326.9	87	225.3	163.4, 303.4	N/A
LP CTX (µg/mmol creatinine)	161	409.1	286.3, 573.2	75	398.7	300.0, 573.2	86	414.4	265.8, 604.8	0.96

	n	Mean	SD	n	Mean	SD	n	Mean	SD	p-value
Δ CTX (z score)				61	-0.14	0.92	61	-0.17	1.11	0.85

Vitamin D insufficient women, baseline 25(OH)D <50nmol/l										
	All			**Placebo**			**Cholecalciferol 1000 IU/day**			**p-value**
	n	Median	IQR	n	Median	IQR	n	Median	IQR	
EP CTX (µg/mmol creatinine)	320	215.8	142.7, 303.2	159	216.0	148.0, 299.6	161	215.7	139.7, 307.5	N/A
LP CTX (µg/mmol creatinine)	335	444.5	293.2, 660.4	176	459.7	333.8, 675.9	159	422.1	249.5, 633.0	<0.01
	n	Mean	SD	n	Mean	SD	n	Mean	SD	p-value
Δ CTX (z score)				126	0.28	0.89	123	-0.15	1.04	<0.01

p-values represent outcomes of tests for differences between the placebo group and vitamin D supplemented group (Rank Sum tests for LP CTX, t tests for Δ CTX (z score)). EP = 14 weeks' gestation; LP = 34 weeks' gestation; ΔCTX = conditional change in CTX (LP CTX adjusted for EP CTX), SD.

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Table 3: Univariate maternal determinants of CTX in early and late pregnancy (Fisher-Yates Scores), standard deviation increase per unit change in maternal predictor, in those with both an early and late pregnancy measure.

Exposure	EP CTX (14 weeks' gestation)				LP CTX [1] (34 weeks' gestation)				Δ CTX [1,3]			
	n	β[2]	95% CI	p-value	n	β[2]	95% CI	p-value	n	β	95% CI	p-value
Maternal age (years)	493	-0.03	(-0.05,-0.01)	<0.001	469	-0.03	(-0.04,-0.01)	0.01	372	-0.02	(-0.037,0.002)	0.07
Strenuous exercise (> 1 hr /week, Y/N)	440	-0.10	(-0.36,0.16)	0.47	425	-0.11	(-0.40,0.18)	0.47	329	-0.17	(-0.50,0.16)	0.31
Smoking in early pregnancy (Y/N)	491	0.36	(0.05,0.68)	0.03	467	-0.20	(-0.53,0.13)	0.24	370	-0.20	(-0.57,0.17)	0.30
Parity (One or more, Y/N)	491	-0.48	(-0.66,-0.31)	<0.001	467	-0.33	(-0.52,-0.15)	<0.001	371	-0.11	(-0.32,0.09)	0.28
Caucasian ethnicity (Y/N)	490	0.17	(-0.25,0.58)	0.44	466	0.23	(-0.21,0.67)	0.31	369	0.33	(-0.20,0.86)	0.22
Weight (kg)	493	-0.01	(-0.01,0.00)	0.07	469	0.002	(-0.004,0.008)	0.50	372	0.01	(-0.001,0.013)	0.12
Height (cm)	488	-0.01	(-0.03,0.00)	0.05	465	-0.02	(-0.03,-0.01)	0.004	368	-0.01	(-0.029,0.004)	0.13
BMI (kg/cm^2)	488	-0.01	(-0.03,0.01)	0.21	465	0.02	(-0.001,0.034)	0.07	368	0.02	(0.001,0.041)	0.04
14 week 25(OH)D (nmol/l)	488	0.004	(-0.001,0.009)	0.14	496	-0.004	(-0.010,0.001)	0.13	371	-0.01	(-0.014,-0.001)	0.03
14 week Calcium (mmol/l)	488	0.53	(-0.56,1.62)	0.34	496	0.67	(-0.36,1.70)	0.20	371	1.14	(-0.09,2.38)	0.07
34 wk. 25(OH)D (nmol/l)					489	-0.001	(-0.016,0.003)	0.55	364	-0.003	(-0.009,0.002)	0.19
34 wk. calcium (mmol/l)					488	0.15	(-0.63,0.92)	0.71	363	0.19	(-0.74,1.11)	0.70
Change in 25(OH)D from 14 to 34 wks. (nmol/l)					487	0.001	(-0.003,0.005)	0.60	363	0.001	(-0.004,0.006)	0.77
Vit D supplemented group (Y/N)					498	-0.18	(-0.35,-0.00)	0.05	372	-0.32	(-0.52,-0.11)	0.002
Season of delivery, summer (proxy for season of measurement)	438	0.02	(-0.16,0.21)	0.81	488	0.07	(-0.11,0.25)	0.44	366	0.07	(-0.14,0.27)	0.52

[1] adjusted for treatment group, except when associations between vitamin D supplemented group and CTX are being tested. [2] standard deviation change in urinary CTX (Fisher Yates Score) per unit exposure. [3] conditional change in CTX (LP CTX adjusted for EP CTX), as Z-score.

Table 4: Associations between EP or LP 25(OH)D, and LP CTX (Fisher-Yates score) or conditional change in CTX (Δ CTX, LP CTX adjusted for EP CTX), SD.

	Exposure	EP 25(OH)D		LP 25(OH)D	
	Outcome	LP CTX[1]	Δ CTX [2]	LP CTX[1]	Δ CTX [2]
	n	486	365	479	358
	β	-0.004	-0.007	-0.004	-0.008
Adjusted for season	95%CI	(-0.010, 0.002)	(-0.013, -0.001)	(-0.008, -0.001)	(-0.012, -0.003)
	p-value	0.156	0.049	0.056	0.001
	n	450	358	443	351
Adjusted for season of delivery, maternal age, parity, BMI, smoking and height	β	-0.003	-0.006	-0.004	-0.008
	95%CI	(-0.009, 0.003)	(-0.013, 0.001)	(-0.008, -0.001)	(-0.012, -0.003)
	p-value	0.396	0.097	0.054	0.002

[1] difference in urinary CTX per unit exposure (Fisher-Yates), SD; [2] conditional change in CTX (LP CTX adjusted for EP CTX), SD.

Table 5: Associations between late pregnancy urine CTX (Fisher-Yates transformed, expressed in standard deviations) or conditional change in CTX (LP CTX adjusted for EP CTX, as Z-score), in the vitamin D supplemented, placebo groups, or combined, and maternal lumbar spine and total hip area, bone mineral content (BMC) and bone mineral density (BMD). Models are adjusted for maternal age, parity, smoking and BMI at baseline.

	Total population				Placebo				Cholecalciferol 1000 IU/day			
LP CTX	**n**	**β**	**95% CI**	**p-value**	**n**	**β**	**95% CI**	**p-value**	**n**	**β**	**95% CI**	**p-value**
Lumbar spine												
Bone area (cm^2)	283	-0.34	(-0.96,0.29)	0.29	135	-0.73	(-1.697,0.235)	0.14	148	-0.08	(-0.92,0.80)	0.86
BMC (g)	283	-1.17	(-2.29,-0.04)	0.04	135	-1.30	(-2.972,0.377)	0.13	148	-1.11	(-2.69,0.46)	0.17
aBMD (g/cm^2)	283	-0.015	(-0.027,-0.003)	0.02	135	-0.009	(-0.028,0.010)	0.34	148	-0.019	(-0.036,-0.003)	0.03
Total hip												
Bone area (cm^2)	279	-0.32	(-0.70,0.06)	0.10	133	-0.32	(-0.94,0.29)	0.30	146	-0.37	(-0.85,0.11)	0.13
BMC (g)	279	-0.68	(-1.21,-0.14)	0.01	133	-0.51	(-1.31,0.30)	0.22	146	-0.87	(-1.62,-0.13)	0.02
aBMD (g/cm^2)	279	-0.012	(-0.02,-0.00)	0.05	133	-0.004	(-0.022,0.013)	0.61	146	-0.017	(-0.033,-0.002)	0.03
Δ CTX	**Total population**				**Placebo**				**Cholecalciferol 1000 IU/day**			
	n	**β**	**95% CI**	**p-value**	**n**	**β**	**95% CI**	**p-value**	**n**	**β**	**95% CI**	**p-value**
Lumbar spine												
Bone area (cm^2)	226	-0.23	(-0.92,0.45)	0.50	109	-0.86	(-1.93,0.22)	0.12	117	0.22	(-0.72,1.17)	0.64
BMC (g)	226	-0.72	(-1.98,0.54)	0.26	109	-1.07	(-2.98,0.84)	0.27	117	-0.66	(-2.49,1.16)	0.47

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aBMD (g/cm^2)	226	-0.01	(-0.02,0.01)	0.22	109	0.00	(-0.03,0.02)	0.78	117	-0.02	(-0.037,0.003)	0.10
Total hip												
Bone area (cm^2)	223	-0.29	(-0.70,0.12)	0.16	108	-0.32	(-0.97,0.33)	0.33	115	-0.34	(-0.87,0.20)	0.21
BMC (g)	223	-0.32	(-0.92,0.29)	0.31	108	-0.24	(-1.15,0.67)	0.60	115	-0.48	(-1.35,0.38)	0.27
aBMD (g/cm^2)	223	0.00	(-0.01,0.01)	0.83	108	0.003	(-0.02,0.02)	0.74	115	-0.01	(-0.03,0.01)	0.47

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Table 6: Associations between conditional change in pregnancy urine CTX (Δ CTX, expressed in standard deviations) in the vitamin D supplemented and placebo groups combined (total population), and neonatal DXA outcomes at the total body and total spine after adjustment for sex, baby age at DXA scan and gestational age at birth.

Δ CTX	Total population			
	n	β	95% CI	p-value
Neonatal total body DXA				
Total area (cm)	321	4.781	(1.567, 7.995)	0.004
Total BMC (g)	321	1.754	(0.798, 2.711)	<0.001
Total BMD (g/cm^2)	321	0.003	(0.001, 0.005)	0.002
Neonatal total spine DXA				
Total spine area (cm)	290	0.140	(0.027, 0.252)	0.015
Total spine BMC (g)	290	0.071	(0.022, 0.120)	0.005
Total spine BMD (g/cm^2)	290	0.004	(0.000, 0.008)	0.038

Δ CTX = conditional change in CTX (LP CTX adjusted for EP CTX), SD.

ORIGINAL UNEDITED MANUSCRIPT

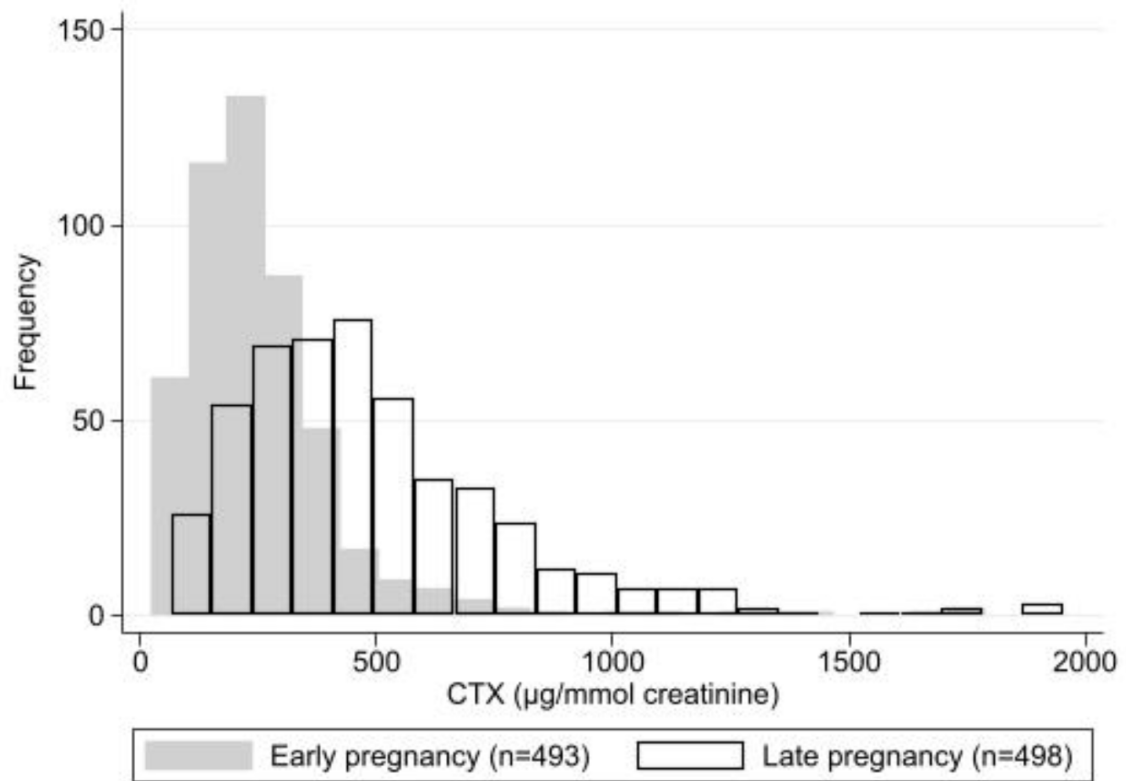**Figure 1**

Frequency distribution of maternal urinary CTX (μg/mmol creatinine) in early (14 weeks' gestation, median 219.4, IQR 154.7, 306.4, n=493) and late pregnancy (34 weeks' gestation; median 437.4, IQR 292.3, 633.0; n=498) in all mothers with a measurement of CTX.
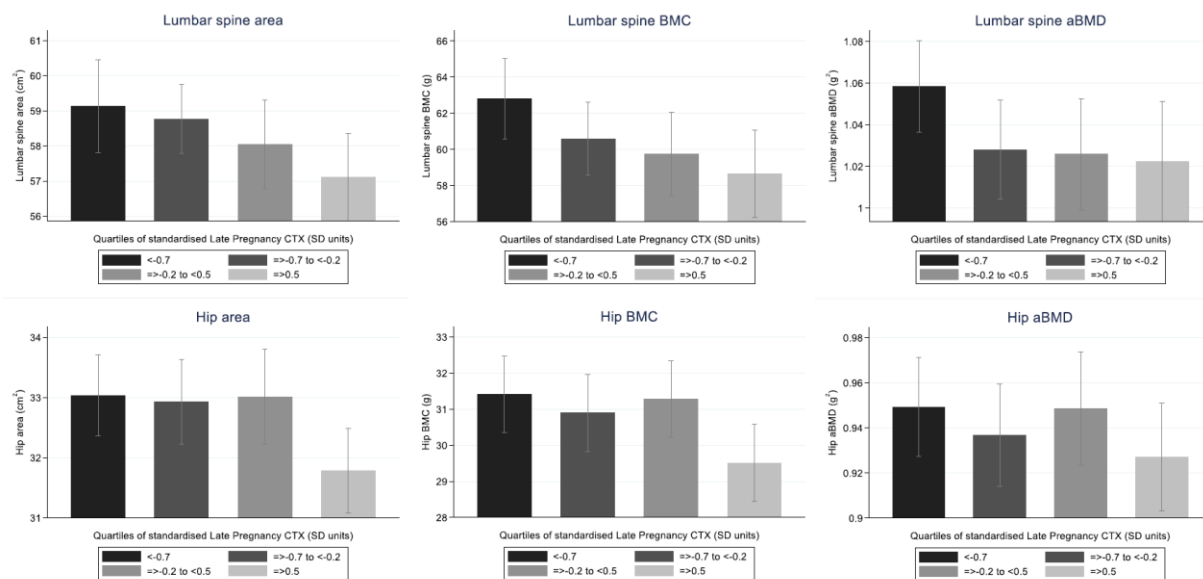
Figure 2

Late pregnancy urinary CTX (shown in quarters of the distribution, SD) and A) maternal postpartum lumbar spine area, BMC and aBMD (n =283) and B) total hip area, BMC and aBMD (n = 279) assessed by DXA (in both groups combined).

CTX divided into quarters of the distribution: -1.5 SD to <-0.7 SD; -0.7 SD to <-0.2 SD, -0.2 SD to <0.5 SD, 0.5 SD or above