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## **Serum Calcium Normal Range in 1,000 Term Newborns**

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HAL Authorization

**Title: Serum calcium normal range in 1,000 term newborns.**

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**Short title: Serum calcium normal range in 1,000 neonates.**

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**Abbreviations:** body mass index (BMI); fibroblast growth factor-23 (FGF23) ; parathyroid hormone (PTH) ; PTH-related protein (PTHrP) ; standard deviation (SD) ; last menstrual period (LMP).

## **Abstract**

**Introduction:** Serum calcium rapidly declines at birth because of the sudden interruption of the maternal-fetal calcium influx. Several factors are known to influence serum calcium in the first days of life, including circulating concentrations of maternal vitamin D. Objective was to establish the normal range variations of neonatal serum calcium according to the current French vitamin D supplementation during pregnancy, i.e., 100,000 IU of cholecalciferol during the third trimester.

**Methods:** We included in our prospective cohort study 1002 mother-newborn dyads recruited from April 2012 to July 2014, in two centers located in the neighborhoods of Paris, France.

**Results:** Total serum calcium at 3 days of life in neonates varied from 2.06 mmol/L to 2.73 mmol/L [2.5 and 97.5 percentiles], with a mean of 2.45 mmol/L. Serum calcium was similar between babies born from vitamin D supplemented mothers and those born from the non-supplemented ones. In univariate and multivariable analyses, we demonstrated the importance of maternal and cord blood 25(OH)D concentrations for newborn serum calcium maintenance.

**Conclusion:** We established that the expected serum calcium in neonates ranges between 2.06 mmol/L and 2.73 mmol/L which is significantly wider than the adult range. This finding should help physicians in the diagnosis of hypo- or hypercalcemia. In addition, our study supports the importance of vitamin D supplementation and 25(OH)D status for neonatal serum calcium maintenance.

## **Introduction**

Bone and mineral metabolism are tightly regulated in humans by several hormones, including parathyroid hormone (PTH), calcitriol, fibroblast growth factor-23 (FGF23), calcitonin, and sex steroids. PTH-related protein (PTHrP) is an additional key player in fetal calcium metabolism[1]. It is the main regulator of the active transport of calcium through the placenta, from the maternal circulation to the fetus, which is necessary for fetal development[1]. In *utero*, human fetuses display serum and ionized calcium that are above maternal levels by 0.30–0.50 mmol/L[2]. These high calcium levels suppress the fetal PTH through the calcium sensing receptor[1]. Fetal calcitriol synthesis is suppressed by elevated calcium and phosphorus and by low PTH levels. Both calcitriol and 25(OH)D pass through the placenta; the latter exhibits a strong correlation between cord and maternal serum values[1,3,4].

Birth triggers a series of physiological changes in mineral homeostasis. First, there is a significant decrease of the serum calcium level in the first day of life due to the sudden interruption of maternal-fetal calcium flux, the suppressed levels of endogenous calcitriol which limits significantly the active transport of calcium through the intestine, and the suppressed PTH during the first 2 days of life[1]. After 48 hours, PTH levels increase significantly, resulting in endogenous neonatal synthesis of calcitriol and increased serum calcium concentration. It is only after a few weeks that the transport of calcium in the intestine is balanced upon the progressive development of the calcitriol-dependent active calcium transport [1,5]. Vitamin D and its metabolites are therefore required for the maintenance of serum calcium level and the prevention of hypocalcemia.

Because of this switch from a maternal-fetal to a neonatal-specific calcium transport, newborns are especially at risk of calcium metabolism disturbances. For instance, hypocalcemia can have life-threatening consequences such as hypocalcemic seizures,

laryngospasm, heart failure and death[5]. Neonatal hypocalcemia is a frequent complication in newborns born to vitamin D-deficient women, affecting 5% of children in the absence of vitamin D supplementation[6,7].

Our team and others have helped show that vitamin D supplementation during pregnancy is associated with higher levels of circulating vitamin D[8,9], improvement of dental enamel formation[10] and intrauterine growth[9], and reduction of preeclampsia[8]. In addition, vitamin D supplementation at recommended doses during pregnancy appears to be safe as it does not modify fetal or neonatal mortality or the frequency of congenital anomalies. In addition, many data support the role of vitamin D in serum calcium maintenance after birth including to limit the risk of hypocalcemia during the few days after birth [2,10–17]. Several guidelines in Europe or in the United States of America recommend vitamin D supplementation during pregnancy[18].

Consequently, the French health authorities have recommended vitamin D supplementation for pregnant women since 1995, through an oral single dose of 100,000 IU during the third trimester. We know from previous studies, including ours, that 25(OH)D concentrations in newborns are significantly higher when the mother receives the supplementation during pregnancy, although 25(OH)D concentrations are mostly observed within the range of vitamin D deficiency[3,19]. The effect of this vitamin D supplementation during pregnancy on the total serum calcium in newborns remains undetermined.

We show here a systematic analysis of total serum calcium levels in 1002 term newborns born to mothers followed up in two French university obstetric centers applying the current recommendations for care.

In addition, we investigated the potential effect of vitamin D supplementation during pregnancy on circulating total serum calcium. Finally, we searched for putative factors associated with changes in total serum calcium in this large sample of newborns.

## **Methods**

### ***Study design***

This study is an ancillary study and used the prospective observational FEPEd cohort of pregnant women in six centers (five French and one Belgian), which was designed to evaluate the relationship between vitamin D status in pregnancy and the risk for preeclampsia[8].

Written informed consent was obtained from each woman before inclusion in the study. This study protocol was reviewed and approved by Assistance Publique-Hôpitaux de Paris ethics committee, approval number [2011/13NICB]. Samples were stored in the Perinat Collection (ANR-10-EQPX-0010). The study was sponsored by the Assistance Publique-Hôpitaux de Paris and was funded by a grant from the Programme Hospitalier de Recherche Publique national 2010 (Ministry of Health-AOM10113).

Briefly, women were included during the first consultation following the diagnosis of pregnancy if they were at between 10 and 14<sup>+6</sup> weeks post last menstrual period (LMP) of a singleton pregnancy. Women were not included in the case of hypercalcemia (serum calcium over 2.65 mmol/L) or any other calcium-phosphorus imbalance. The skin phototype was determined according to the Fitzpatrick skin type classification [20] and the geographical origin was self-declared. Information on socio-economic status, or on the ingestion of other vitamin D-containing supplements were not available. Further documentation can be found in Courbebaisse et al. and Benachi et al.[3,8].

### ***Participants and data collection***

To evaluate the impact of the French recommendations on neonatal total serum calcium, we excluded the Belgium recruiting center and kept two recruiting centers located in Paris

neighborhoods (AP-HP, Antoine Bécère and Bicêtre, Saclay University Hospitals) that included mother-newborn dyads from April 2012 to July 2014. Mothers received a prescription for an oral bolus vitamin D dose (100,000 IU of cholecalciferol) at the 7th month of pregnancy according to current French recommendations[19]. They were asked at 34 weeks post LMP whether they received or not the vial. Dyads were included if the delivery occurred at 36 weeks post LMP and thereafter.

In addition to the data collected during the pregnancies, a blood sample of 250  $\mu$ L was collected by venipuncture from the newborns in the maternity hospital at 3 days of life for serum calcium measurement, at the time of neonatal screening.

Newborn characteristics were recorded, including sex, weight, length, and head circumference at birth. We used the revised Fenton growth chart and PediTools to calculate standard deviation scores for weight, length, and head circumference at birth[21,22].

All blood samples were centrifuged within one hour of sampling. Total serum calcium was measured locally by a colorimetric assay with the Ortho VITROS 5.1 FS automated chemistry system (Ortho Clinical Diagnostics) . Serum aliquots were stored locally at  $-20^{\circ}\text{C}$  and transferred monthly to the department of Physiology of Necker University hospital (Paris, France) where 25-OHD levels were measured in duplicate by radioimmunoassay (RIA) after pretreatment with acetonitrile to allow the denaturation of any protein that may interfere in the assay (Diasorin, Stillwater, MN, USA) [23]. This method was based on an antibody that was cospecific for 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> and did not cross-react with the 25OHD C<sub>3</sub>-epimer which is largely present infants. In our hands, intra- and inter-assay CVs were <10 % over a large range of concentrations (2.5-100 ng/mL). When the measurements were performed, this assay had been recently calibrated against the NIST reference material. Quality control was ensured through the participation of this laboratory in the DEQAS quality assurance program and in the French national control of quality.

### *Statistical analysis*

Statistical analysis was performed using R software (Vienna, Austria)[24]. Results were expressed as mean  $\pm$  standard deviation (SD) for quantitative variables, and as number of patients and percentages for qualitative variables.

Comparisons of mother-newborn dyads depending on 7th month vitamin D supplementation were made for quantitative variables by Student's t-test and for qualitative variables by a chi-square test when feasible, or Fisher's exact test when not.

Factors associated with newborns' total serum calcium levels were studied by linear regression. Factors with a p-value lower than 10% or known to be associated with serum calcium were studied together in a multivariable linear regression model. In this model, all the variance inflation factors are low. Associations between quantitative values were studied using Pearson correlation coefficients.

All tests were two-sided, with a significance level of  $p < 0.05$ .

## Results

### *Demographics and clinical characteristics of the patients*

From the initial FEPED cohort, 1699 mother-newborn dyads came from the two hospitals included in the study (**Figure 1**). Seventy-nine were excluded because gestational age at delivery was below 36 weeks post LMP, 82 and 536 because of clinical or serum calcium measurements missing data respectively. 1002 mother-newborn dyads were included in this study. There are only slight differences when comparing the full cohort of dyads with the cohort of included dyads. The maternal age was slightly higher in included dyads than in non-included (31.6 vs. 30.6 ;  $p=0.001$ ); the BMI before pregnancy (24.1 vs. 23.5 ;  $p=0.017$ ), the season of conception (50.1% vs. 41.2% ;  $p=0.001$ ) and the gestational age (39.5 vs. 39.7 ;  $p=0.011$ ) differed between the 2 cohorts. Therefore, the whole study shows the findings from the 1002 mother-newborn dyads.

The mean age of the mothers at inclusion was  $31.6 \pm 5.1$  years (**Table 1**). Before pregnancy, the mean BMI was  $24.1 \pm 4.9$  kg/m<sup>2</sup>. Nearly 80% of women had a skin phototype between I and IV according to Fitzpatrick's scale. More than half of the women came from metropolitan France. Before pregnancy, 15.8% of mothers were smokers, whereas at the time of inclusion (between 10- and 15-week post LMP), only 2.1% were still smoking.

The mean gestational age was  $39.1 \pm 1.4$  week post LMP at delivery, which was mainly vaginal (76.3%). The newborns (52.8% boys) had a mean weight, length, and head circumference at birth of  $3,310 \pm 498$  g ( $-0.23 \pm 0.93$  SD),  $49.2 \pm 2.3$  cm ( $-0.48 \pm 0.85$  SD), and  $34.5 \pm 1.5$  cm ( $-0.09 \pm 1.01$  SD), respectively.

90.9% of women ingested the 100,000 IU of cholecalciferol supplementation as recommended in France around the 7th month of pregnancy. 2.9% of mothers had vitamin D supplementation during the first trimester, and 0.8% took calcium supplementation during pregnancy. At the maternity hospital, the mean total serum calcium measured in newborns at 3 days of life was  $2.45 \pm 0.16$  mmol/L (**Figure 2**). The 2.5 and 97.5 percentiles were 2.06 mmol/L and 2.73 mmol/L, respectively. About 12.9% of the newborn values were above 2.60 mmol/L (adult local upper normal range) and 6.1% were below 2.20 mmol/L (adult local lower normal range).

#### ***Comparison of mother-newborn dyads depending on 7th month vitamin D supplementation***

As expected, mothers who received the cholecalciferol supplementation had significantly higher 25(OH)D concentrations during the third trimester of pregnancy than non-supplemented ones, i.e.,  $79.1 \pm 29.2$  nmol/L and  $66.6 \pm 30.5$  nmol/L, respectively ( $p < 0.001$ , **Table 2**). Similarly, 25(OH)D concentrations were higher in cord blood from supplemented mothers than in non-supplemented ones, i.e.,  $41.4 \pm 18.2$  nmol/L and  $35.9 \pm 17.0$  nmol/L, respectively ( $p = 0.022$ , **Table 2**).

At birth, babies born to supplemented mothers had similar weight, length, and head circumference than those born to non-supplemented mothers. In addition, total serum calcium at 3 days of life was similar in children born to supplemented or non-supplemented mothers, i.e.,  $2.42 \pm 0.16$  mmol/L and  $2.45 \pm 0.16$  mmol/L,  $p = 0.563$ , respectively (**Figure 3**).

#### ***Univariate analysis for factors influencing newborns' serum calcium***

In the univariate model, serum calcium was significantly associated with third trimester maternal and neonatal cord blood 25(OH)D concentrations ( $p < 0.01$  and  $p < 0.001$ ,

respectively, **Table 3**). Vitamin D supplementation before inclusion or during the third trimester of pregnancy did not influence neonatal total serum calcium.

Through this analysis, we identified gestational age at delivery ( $p < 0.001$ ), delivery mode ( $p < 0.001$ ), newborns' sex ( $p < 0.001$ ) and mothers' BMI before pregnancy ( $p < 0.001$ , **Table 3**) as determinants of neonatal serum calcium. However, the season of conception, the skin phototypes, and the geographical origin of the dyads were not associated with the neonatal calcium concentrations.

#### *Multivariable analysis for factors influencing serum calcium in newborns*

Through the multivariable analysis we confirmed that gestational age at delivery ( $p = 0.002$ ), newborn sex ( $p < 0.001$ ) and 25(OH)D at cord blood ( $p < 0.001$ ) appeared to be associated with neonatal serum calcium (**Table 4**). We also confirmed the lack of association between maternal vitamin D supplementation and total serum calcium in newborns.

#### *Correlation between 25(OH)D in cord blood and serum calcium in newborns*

A positive correlation was found between neonatal cord blood 25(OH)D concentrations and serum calcium at 3 days of life ( $r = 0.19$ ,  $p < 0.001$ , shown in **Fig. 4**).

## Discussion

We report the first study describing total serum calcium levels in healthy newborns born at  $\geq$  36 weeks post LMP to women managed according to current French vitamin D supplementation recommendations. Both in univariate and multivariable analysis, and in contrast with other factors, vitamin D supplementation before inclusion or during the third trimester of pregnancy was not associated with neonatal total serum calcium.

Mean total serum calcium in term newborns measured at 3 days of life was  $2.45 \pm 0.16$  mmol/L, 2.5% of the values being below 2.06 mmol/L and above 2.73 mmol/L respectively. We did not undertake a sample size calculation; however, the precision of this estimate calculated a posteriori is 0.024 mmol/L, which is very good. In adults, the normal range for serum calcium is between 2.15 mmol/L and 2.60 mmol/L[25–27]. Our study confirms that total serum calcium concentration is higher in newborns; therefore it may vary with age[28].

It is of major importance to determine the reference for serum calcium in newborns, as the narrowed adult range may lead to over-diagnosis of both hypo- and hypercalcemia, as demonstrated by the 12.9% and 6.1% of neonatal serum calcium values above 2.60 mmol/L and below 2.20 mmol/L, respectively. Our results are consistent with those of Roizen et al., who found a serum calcium reference interval of 1.8 mmol/L to 2.8 mmol/L from birth to 90 days (colorimetric assay with the Ortho VITROS 5.1 FS (Ortho Clinical Diagnostics))[29], but are higher than those of Tietz et al. who report an interval of 1.9 mmol/L to 2.6 mmol/L between 0 and 10 days (spectrophotometric measurements)[25]. We are aware that our study is limited by factors that interfere with the calcium measurement, e.g., pH, protein concentration or venipuncture technique[30,31].

There is much evidence of the role of vitamin D supplementation in the prevention of neonatal hypocalcemia[3,9,10]. We have shown previously in this cohort that 25(OH)D levels during the pregnancy, especially during the 3<sup>rd</sup> trimester, correlates with cord blood 25(OH)D levels[3]. Noteworthy, the current supplementation regimen recommended for French pregnant women is not sufficient to reach optimal target vitamin D levels at birth in the offspring. We could not show in this prospective cohort through univariate and multivariable analyses that vitamin D supplementation influences neonatal total serum calcium. This may be due to the limited statistical power of our study by the high proportion of women who received vitamin D during their pregnancy (90.9%). It is possible that one single dose of 100,000 IU of cholecalciferol[32] is insufficient to prevent vitamin D deficiency in most newborns[3]. Noteworthy, in this study where most mothers were vitamin D supplemented, the reference range of serum calcium have been determined in a large cohort of term newborns having, except for 47 babies, a 25(OH)D concentration below 75 nmol/l. In addition, the serum calcium was measured at day 3 of life in our study, well before the active vitamin D dependent transport of calcium through the gut may occur[5].

There is huge variation among countries in vitamin D supplementation recommendations. For instance, Sweden and the UK recommend 400 IU/day during pregnancy; Spain and USA recommend 600 IU/day; whereas Belgium and Germany recommend 800 IU/day[32–34]. We can hypothesize that both the dose and the mode of vitamin D administration (stoss therapy[35] vs. daily dose) may account for the lack of correlation between neonatal serum calcium and vitamin D during pregnancy[18,36].

Finally, we showed that 25(OH)D at cord blood was associated with neonatal serum calcium, and we cannot exclude that 25(OH)D levels are more relevant in determining serum calcium than whether supplementation was received. Indeed, both the univariate model analysis and the correlation analysis supported the significant association of neonatal serum calcium with

cord blood 25(OH)D concentrations ( $r = 0.19$ ,  $p < 0.001$  and  $p < 0.001$  respectively). Data regarding the association between cord blood 25(OH)D concentrations and serum calcium in term neonates are scarce. Reported cohorts are smaller than ours, and often compare vitamin D supplemented to non-supplemented mothers. Except for a weak association in vitamin D supplemented mothers, these reports failed to identify a correlation between these 2 factors [12,37].

We found several factors influencing neonatal serum calcium, such as gestational age at delivery, newborn sex, and 25(OH)D at cord blood. However, other factors such as BMI, skin phototypes and geographical origin were not associated with neonatal calcium concentrations in this study, whereas they are known to impact maternal 25(OH)D concentrations[38].

## **Conclusion**

This large prospective cohort of more than 1,000 dyads is the first to establish the expected range of neonatal serum calcium according to current vitamin D supplementation practice for pregnant women. Serum calcium is often checked in newborns because of the nonspecific clinical manifestations of both hypo- and hypercalcemia. Identification of low or elevated serum calcium levels in newborns triggers investigations and sometimes treatment. It is therefore critical to rely on accurate reference ranges of serum calcium physiological variations. In addition, our study confirms the importance of vitamin D supplementation and 25(OH)D status for maintenance of neonatal serum calcium levels.

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**Statement of Ethics:** Written informed consent was obtained from each woman before inclusion in the study. The protocol was conducted in accordance with the Declaration of Helsinki and was approved by a local independent Ethics Committee (2011/13NICB). It is registered with the ClinicalTrials.gov (identifier NCT01648842).

**Conflict of Interest Statement:** The authors have no conflicts of interest relevant to this article to declare.

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#### **Author contribution statement**

Prs Linglart, Benachi and Gajdos conceptualized and designed the study, drafted the initial manuscript, and reviewed and revised the manuscript.

Dr Letamendia, Boithias and Lambert conceptualized and designed the study, and reviewed and revised the manuscript.

Dr Ouaras-Lounis, Prs Théron and Souberbielle designed the data collection instruments, collected data, and reviewed and revised the manuscript.

Dr Levallant L. drafted the initial manuscript, and reviewed and revised the manuscript.

Dr Levallant M. performed statistical analyses, drafted the initial manuscript, and reviewed and revised the manuscript.

**All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.**

**Data Availability Statement:** All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author..

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## Figures and tables legends

**Figure 1:** Flow chart of the patients included in the study.

**Figure 2:** Distribution of serum calcium in newborns at 3 days of life.

**Figure 3:** Serum calcium in newborns at 3 days of life depending on maternal vitamin D supplementation.

**Figure 4A:** Correlation between 25(OH)D level measured during the 2<sup>nd</sup> trimester and 25(OH)D level measured in cord blood; correlations were calculated in 25(OH)D-supplemented mothers (dashed line), n= 881 (r = 0.63; 95% CI [0.58;0.67]; p <0.001) and in non-supplemented mothers (dotted line), n= 88 (r = 0.69; 95% CI [0.50;0.82]; p <0.001). **4B:** Correlation between serum calcium at 3 days of life in newborns and 25(OH)D level measured in cord blood (r = 0.19; 95% CI [0.12;0.26]; p <0.001).