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25 **Keywords:** serum calcium; vitamin D; reference values; newborns; pregnancy.

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33

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36

37 **Clinical Trial Registration number:** NCT01648842.

38 **Abstract**

39 **Introduction:** Serum calcium is frequently measured during the neonatal period, and is
40 known to be influenced by the vitamin D status. We hypothesized that the 25OHD
41 concentration may influence the lower limit of the serum calcium normal range in neonates.

42 **Methods:** We included in our prospective cohort study 1002 mother-newborn pair recruited
43 from April 2012 to July 2014, in two centers located in the neighborhoods of Paris, France,
44 whose serum calcium was measured at 3 days of life. We established, after exclusion of
45 outliers, a 95% confidence interval (CI) for serum calcium 1) in our whole population of 1002
46 neonates, 2) in neonates with a cord blood 25OHD concentration ≥ 30 nmol/L, and 3) in
47 those with a 25OHD ≥ 50 nmol/L.

48 **Results:** The mean serum total calcium was 2.46 ± 0.13 mmol/L [95% CI: 2.19-2.72 mmol/L],
49 2.47 ± 0.25 mmol/L [95% CI: 2.22-2.72 mmol/L], and 2.50 ± 0.25 mmol/L [95% CI: 2.25-2.75
50 mmol/L] in the whole group, in the 514 neonates with 25OHD ≥ 30 nmol/L, and in the 202
51 neonates with 25OHD ≥ 50 nmol/L respectively. The lower limit of the 95% range was
52 significantly higher in neonates with 25 OHD ≥ 30 nmol/L ($p < 0.05$) and ≥ 50 nmol/L ($p < 0.001$)
53 than in the entire cohort.

54 **Conclusion:** We show that the lower limit of the normal serum calcium range is higher in
55 groups with a higher 25OHD than in unselected subjects. We propose that the reference
56 range for serum calcium in neonates is 2.25 to 2.75 mmol/L.

57 **Introduction**

58 Serum calcium is frequently measured during the neonatal period, even in newborns who
59 have no specific symptoms of hypo- or hypercalcemia. This is done for example in case of
60 prematurity, low birth weight or gestational diabetes mellitus (1). Establishing a specific
61 normal range for serum calcium in neonates is thus of paramount importance to avoid
62 misdiagnosis of hypo- or hypercalcemia, which could lead to unnecessary further
63 examinations and follow-up. We recently published the results of serum total calcium range
64 in more than 1000 healthy neonates at 3 days of life (2). As the distribution of serum calcium
65 was not gaussian in this population, we used the non-parametric method to calculate the
66 normal range which varied from 2.06 mmol/L to 2.73 mmol/L [2.5 and 97.5 percentiles], with
67 a mean of 2.45 mmol/L and a median of 2.45 mmol/L. However, in this study, we included all
68 the cohort and did not take the vitamin D status into account, whereas it is well-known to
69 influence both calcium and phosphorus homeostasis. Hypovitaminosis D, defined by a serum
70 25-hydroxyvitamin D (25OHD) concentration < 50 nmol/L, is common in French pregnant
71 women (3,4) and children (5). The binding of calcitriol, the active metabolite of 25OHD, to
72 the vitamin D receptor elicits the expression of calcium transporters in the gut, thus allowing
73 the absorption of calcium. Insufficient levels of 25OHD lead to a reduced calcium absorption
74 and hypocalcemia (6,7). Several authors have reported lower serum calcium concentrations
75 in vitamin D deficient neonates in comparison to those with normal vitamin D concentrations
76 (6). As vitamin D deficiency is an unwanted condition with possible harmful consequences
77 such as the increased risk of hypocalcemia, it is relevant to exclude vitamin D deficient
78 subjects from a population of neonates recruited to establish reference values for serum
79 calcium. This has previously been proposed by Roizen et al. who reported data from a large
80 cohort of children of various ages showing that the lower limit of normal of serum calcium is

81 higher in their vitamin D-repleted children than in those with vitamin D insufficiency and
82 deficiency (8).
83 According to these results, we therefore hypothesized that the 25OHD status may influence
84 the serum total calcium normal range in our cohort of 1002 healthy neonates. To test this
85 hypothesis, we used the same protocol to establish a 95% confidence interval for serum
86 calcium 1) in the whole population of 1002 neonates, i.e., without any selection according to
87 their 25OHD serum level, 2) in neonates with a serum 25OHD concentration ≥ 30 nmol/L, a
88 threshold that defines severe vitamin D deficiency, and 3) in neonates with a serum 25OHD
89 concentration ≥ 50 nmol/L which is the definition of vitamin D sufficiency.

90

91 **Material and methods**

92 ***Study design***

93 This study is an ancillary study of the prospective observational FEPED cohort of pregnant
94 women, which was designed to evaluate the relationship between vitamin D status in
95 pregnancy and the risk for preeclampsia (9). Written informed consent was obtained from
96 each woman before inclusion in the study. The study protocol was conducted in accordance
97 with the Declaration of Helsinki and was reviewed and approved by Assistance Publique-
98 Hôpitaux de Paris (AP-HP) ethics committee, approval number [2011/13NICB]. The study was
99 sponsored by the AP-HP and was funded by a grant from the Programme Hospitalier de
100 Recherche Publique national 2010 (Ministry of Health-AOM10113). It is registered with the
101 ClinicalTrials.gov identifier NCT01648842.

102 Briefly, women were included during the first consultation following the diagnosis of
103 pregnancy if they were at between 10 and 14 weeks post last menstrual period (LMP) of a
104 singleton pregnancy. Women were not included in the case of hypercalcemia (serum calcium

105 over 2.65 mmol/L) or any other calcium-phosphorus imbalance. Further documentation can
106 be found in Courbebaisse et al. (3), Benachi et al. (3,9) and our recent report (2).

107 ***Participants and data collection***

108 We used the samples obtained during the FEPED study in two recruiting centers located in
109 Paris neighborhoods (AP-HP, Antoine Bécère and Bicêtre, Saclay University Hospitals) that
110 included mother-newborn pair from April 2012 to July 2014. Mothers received a prescription
111 for an oral bolus vitamin D dose (100,000 IU vitamin D3) at the 7th month of pregnancy
112 according to current French recommendations (10). They were asked at 34 weeks post LMP
113 whether they received or not the vial. Pairs were included if the delivery occurred at 36
114 weeks post LMP and thereafter.

115 Cord blood for the measurement of 25OHD was available for all cases included in the present
116 study. In addition to the data collected during the pregnancies, a blood sample of 250 µL was
117 collected by venipuncture from the newborns in the maternity hospital at 3 days of life for
118 serum calcium measurement, at the time of neonatal screening.

119 All blood samples were centrifuged within one hour of sampling. Serum total calcium was
120 measured locally by a colorimetric assay with the Ortho VITROS 5.1 FS automated chemistry
121 system (Ortho Clinical Diagnostics). Serum aliquots were stored locally at -20°C and
122 transferred monthly to the department of Physiology of Necker University hospital (Paris,
123 France) where 25OHD levels were measured in duplicate by radioimmunoassay (RIA) after
124 pretreatment with acetonitrile to allow the denaturation of any protein that may interfere in
125 the assay (Diasorin, Stillwater, MN, USA) (11). This method was based on an antibody that
126 was cospecific for 25OHD2 and 25OHD3 and did not cross-react with the 25OHD C3-epimer
127 which is largely present in infants. In our hands, intra- and inter-assay CVs were <10 % over a
128 large range of concentrations (2.5-100 ng/mL). When the measurements were performed,

129 this assay had been recently calibrated against the NIST reference material. Quality control
130 was ensured through the participation of this laboratory in the DEQAS quality assurance
131 program and in the French national control of quality.

132 ***Statistical analysis***

133 Statistics were performed using R software (Vienna, Austria) (12). Results for quantitative
134 variables were expressed as mean \pm standard deviation (SD) or median (inter-quartile range
135 (IQR)) according to the gaussian or non-gaussian distribution of the variable, and as number
136 of patients and percentages for qualitative variables. Correlation between cord blood 25OHD
137 concentration and serum calcium at 3 days of life was assessed by simple regression. To
138 determine the 95 % serum total calcium range in 3 groups of neonates that we considered
139 according to their 25OHD level (1: all subjects, 2: 25OHD \geq 30 nmol/L, and 3: 25OHD
140 concentration \geq 50 nmol/L), we used the Horn method (13). We first detected outliers,
141 defined as serum calcium concentrations below [quartile 1 (Q1) - 1.5 x IQR] and above
142 [quartile 3 (Q3) + 1.5 x IQR], after log transformation of the raw values. We then tested the
143 normality of the remaining values (Shapiro-Wilk normality test) and we calculated the mean
144 \pm 2 SD to define the 95% range in case of normal distribution or we used the non-parametric
145 method in case of non-gaussian distribution. A p-value $<$ 0.05 was considered significant. The
146 Welch Two Sample t-test was used to compare 95% intervals.

147

148 **Results**

149 Among the whole group of 1002 cases, 911 (90.9%) of the mothers ingested the 100,000 IU
150 vitamin D3 dose around the 7th month of pregnancy. Serum 25OHD concentrations were
151 higher in cord blood from neonates of supplemented mothers than in non-supplemented
152 ones, 37.4 nmol/L (IQR 25.0) and 34.9 nmol/L (IQR 25.0), respectively (p=0.022). Serum

153 calcium at 3 days of life was significantly associated with neonatal cord blood 25OHD
154 concentrations ($r=0.19$; $p < 0.001$). After excluding 39 outliers (6 high values and 33 low
155 values) mean and median serum calcium were 2.46 ± 0.13 nmol/L and 2.46 nmol/L (IQR
156 0.19) respectively. As the distribution of the remaining 963 serum calcium was non-gaussian,
157 we used the non-parametric method to determine the 95% confidence interval of serum
158 calcium which was 2.19-2.72 mmol/L (2.5-97.5th percentile).

159 Five hundred and thirty-two neonates (53.1% of the whole group) had a cord blood 25OHD \geq
160 30 nmol/L. After excluding 18 outliers (14 high values and 4 low value) and verification that
161 the distribution of the remaining 514 calcium measures was normal, the mean \pm 2 SD serum
162 total calcium measured at 3 days of life was 2.47 ± 0.25 mmol/L, corresponding to a 95%
163 interval of 2.22 to 2.72 mmol/L.

164 Two-hundred and eight neonates (20.8% of the whole group) had a cord blood 25OHD \geq 50
165 nmol/L. After excluding 6 outliers (5 high values and 1 low value) and verification that the
166 distribution of the remaining 202 serum calcium was normal, the mean \pm 2 SD serum total
167 calcium measured at 3 days of life was 2.50 ± 0.25 mmol/L, corresponding to a 95% interval
168 of 2.25 to 2.75 mmol/L.

169 The above results are summarized in Table 1. According to the Welch Two Sample t-test, the
170 lower limit of the 95% range was significantly higher in neonates with cord blood \geq 30
171 nmol/L ($p < 0.05$), and in those with 25OHD \geq 50 nmol/L ($p < 0.001$) than in the entire cohort.

172

173 **Discussion**

174 In the present study, we assessed ranges of serum calcium concentration in groups of healthy
175 neonates at 3 days of life selected according to their vitamin D status. We show that the
176 lower limit of these ranges is dependent on the cord blood 25OHD level of the neonates, and

177 is higher in groups with a higher 25OHD concentration than in unselected subjects. This
178 supports the idea to include only vitamin D-repleted neonates in a population recruited to
179 establish reference values for serum calcium.

180 Like in the present study, several authors have reported a significant correlation between
181 serum 25OHD and calcium levels in neonates and/or a lower serum calcium in neonates with
182 low 25OHD concentration (14–18). Roizen et al. tested whether reference intervals for serum
183 total calcium in a pediatric population (0 to 19 years) were different in vitamin D-replete
184 children and in children unselected for their vitamin D status (8). Like in the neonates in the
185 present study, they found that at all ages, the lower limit, but not the higher limit, of the
186 reference intervals for normal subjects with vitamin D sufficiency, i.e., 25OHD levels ≥ 50
187 nmol/L, were significantly higher than those for unselected subjects. They found a reference
188 interval for serum calcium of 2.0-2.8 mmol/L in their group of children aged 0-90 days with a
189 25OHD serum concentration between 50 and 200 nmol/L. It is worth noting that the lower
190 limit of this range is clearly lower than the one we characterized, i.e., 2.25 mmol/L, in our
191 cohort of neonates with a 25OHD ≥ 50 nmol/L. We suggest several possible explanations for
192 this finding. First, the cohort of Roizen et al. was made of hospitalized children, many of
193 them being acutely ill. Although none of their patients were known as having an anomaly of
194 the calcium and phosphorus homeostasis, it remains possible that some patients had
195 asymptomatic moderate hypocalcemia. Second, contrary to Roizen et al., we identified and
196 excluded a certain number of outliers from our groups of subjects. This had a significant
197 influence on the lower limit of the ranges of serum calcium which was 2.06 mmol/L in the
198 whole cohort of 1002 unselected neonates (2), and 2.19 mmol/L in the selected cohort of
199 963 neonates after exclusion of 39 outliers. Excluding outliers from a population recruited to
200 establish the reference values of a given biological parameter may be critical and has direct

201 consequences on decision taking, thus patients' management. Third, Roizen et al. did not
202 focus on the first few days after birth, a period at high risk of disturbances in calcium
203 homeostasis due to the physiological changes occurring at birth, that includes the brutal
204 interruption of the calcium flux from the cord blood and the need of a reliable digestive
205 absorption of calcium. Finally, Roizen et al. found that children with a 25OHD concentration
206 above 75 nmol/L had a higher serum calcium than those with a 25OHD level between 50 and
207 75 nmol/L suggesting that 75 nmol/L was a more relevant threshold than 50 nmol/L to define
208 vitamin D-replete children. Our analysis was limited by the number of neonates with a cord
209 blood 25OHD \geq 75 nmol/L (n=47; 0.05% of the entire cohort) which did not allow us to test
210 this hypothesis.

211 Some limitations of this study are that we only measured serum calcium, without taking into
212 account factors that may interfere with this calcium measurement (for example, pH, protein
213 concentration or venipuncture technique (19,20)). We were also unable to measure free
214 25(OH)D or other parameters of calcium metabolism due to the small volume of the blood
215 samples.

216 In conclusion, we propose to include only vitamin D-replete neonates in a population
217 intended to establish reference values for the serum calcium concentration. This has a strong
218 influence on the reference range, leading to a narrowing of the reference interval, especially
219 by increasing the lower value. Given our findings, we suggest that the reference range for
220 serum calcium in neonates is 2.50 ± 0.25 mmol/L [95% interval of 2.25 to 2.75 mmol/L].

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225 management and storage of biospecimens.

226

227 **Data Availability Statement**

228 The data that support the findings of this study are available from the corresponding author,
229 upon reasonable request.

230

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289

290 **Table 1:** 95% confidence range for serum calcium (2.5-97.5 percentile) in 3 groups of
 291 neonates selected according to their vitamin D status. The lower limit of these range
 292 increases with increasing vitamin D status of the group.

293 *: In these cases, median and mean were the same

	n	Serum calcium after exclusion of outliers. Mean ± SD; (IQR)	95% confidence interval for serum calcium (2.5-97.5 percentile)
Whole cohort of neonates unselected according to their vitamin D status	963	*2.46 ± 0.13 nmol/L; (0.19)	2.19-2.72 mmol/L
Neonates with a serum 25OHD ≥ 30 nmol/L	532	*2.47 ± 0.13 nmol/L; (0.18)	2.22-2.72 mmol/L
Neonates with a serum 25OHD ≥ 50 nmol/L	208	*2.50 ± 0.13 nmol/L; (0.18)	2.25-2.75 mmol/L

294