**Title:** Neonatal Vitamin D Status from Archived Dried Blood Spots and Future Risk of Fractures in Childhood – results from the D-tect study, a Population-Based Case-Cohort Study

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**Keywords:** fractures, vitamin D, dried blood spots, epidemiology, osteoporosis, development

**Abbreviations:** Avon Longitudinal Study of Parents and Children (ALSPAC); Confidence interval (CI); Dual-energy x-ray absorptiometry (DXA); Haematocrit (HCt); Liquid chromatography tandem mass spectroscopy (LC-MS/MS); National Patient Registry (NPR); Odds ratio (OR).

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**Abstract**

**Background:** Whether antenatal and neonatal vitamin D status have clinical relevance in fracture prevention has not been examined extensively, although observational studies indicate that fetal life may be a sensitive period in relation to bone growth and mineralization during childhood.

**Objective:** To examine if 25-hydroxyvitamin D3 (25(OH)D3) concentrations in stored neonatal dried blood spot (DBS) samples are associated with pediatric fracture risk. We hypothesized that in particular low neonatal vitamin D status may be a risk factor for fracture incidence among children.

**Design:** In a register-based case-cohort study design, the case group composed of 1,039 individuals, which were randomly selectedfrom a total of 82,154 individuals, who were born in 1989-99 and had been admitted to a Danish hospital with a fracture of the forearm, wrist, scaphoid bone, clavicle or ankle at the age 6-13 years. The subcohort composed of 1600 individuals randomly selected from all Danish children born during 1989-99. The **N**eonatal 25(OH)D3 concentrations in DBS were assessed using highly sensitive chromatography-tandem mass spectrometry.

**Results:** The mean (SD)25(OH)D3 concentrations for all subjects was 27.7 (18.9) nmol/L (median, 23.5; IQR, 13.3, 37.3), and showed significant monthly variation (p<0.0001) with highest values in July and August. The middle quintile of neonatal 25(OH)D3 had lower odds for sustaining a fracture compared to the lowest quintile (adj. OR: 0.75; 95% CI: 0.58, 0.96), but a global test did not show any significant overall association (adj. p-value: 0.13).

**Conclusion:** This study suggested that neonatal vitamin D status does not influence subsequent fracture risk in childhood. This in accordance with studies that report no association between antenatal maternal vitamin D status and childhood fractures. Further studies are needed to examine fracture risk in relation to prenatal vitamin D status in a randomized controlled setting.

**Introduction**

Globally, the fracture incidence of forearm, hand and foot is high among healthy children, but varies across countries, age, gender and site (1-5). Multiple factors are contributing to the increased risk of pediatric fractures, such as low bone mass, socioeconomic status, childhood obesity and lifestyle, i.e. physical activity (risk-taking behavior), and diet composition (6-8). However whether antenatal and neonatal vitamin D status has clinical relevance in fracture prevention has not been examined extensively, as only few studies have related maternal vitamin D status to offspring fracture risk and found no association (9;10). However, in a sensitivity analysis in the Danish Fetal Origins 1988 cohort among 850 mother-offspring pairs, Petersen et al. found a borderline inverse association between 25(OH)D as a continuous variable and offspring forearm fractures (p=0.054), but there was no association when 25(OH)D concentrations were categorized according to clinical cutoff values (9).

On the other hand, observational studies have examined intrauterine vitamin D status (or proxy measures of vitamin D) and long-term bone growth and density among offspring, finding either a direct (11-13) or no association (14). The studies were primarily carried out in Caucasian children; however the studies differentiated in several respects, such as the study design, the vitamin D assessment method, the geographical latitude of the country of the study population, and age range of the offspring at the outcome measurements.

To our knowledge, no studies have examined neonatal vitamin D status in relation to pediatric fracture risk, yet in Denmark, we have optimal opportunities for studying such associations, through the access to comprehensive and complete national health registers with long follow-up, combined with systematically collected biological material.

Recently, 25(OH)D measured from dried blood spots (DBS), collected in the context of the American or European newborn screening programs for phenylketonuria, have been used to examine associations between neonatal 25(OH)D concentration and development of numerous other outcomes such as schizophrenia (15), childhood brain tumor risk (16), type 1 diabetes (17;18), multiple sclerosis (19;20), autism (21), inflammatory bowel disease (22) and cardiovascular disease risk markers (23), but measured neonatal 25(OH)D concentrations from DBS has not previously been compared among pediatric fracture cases and controls.

We used a case-cohort sampling design to compare 25(OH)D3 concentrations in neonatal dried blood samples retrieved from the Danish Biological Specimen Bank for Neonatal Screening for individuals who developed fractures and a random subcohort.

# Methods

## PARTICIPANTS

The Danish Civil registration system was used to identify all live-born children from 1981 and 2002 (n= 1,360,466) and a random subcohort thereof was sampled for 25(OH)D analyses to be used across the D-tect studies (n=3,585) (24). Individuals from twin or multiple births were allowed in the sample. The Danish National Patient Registry (NPR) was used as source to assess fracture outcome. The NPR is a mandatory nationwide health register, which was established in 1977. There have been important changes over time in the information reported to the NPR and most importantly for our data, in 1995, the registry started to include information on outpatients and patients from emergency rooms (25), while up until this day, only hospitalized fracture cases were registered mandatorily. The consequences were a substantial increase in numbers of fracture cases registered from this day on. To avoid this sudden increase in registered fracture cases, in combination with a pre-specified age group of interest (age 6-13 years), we only selected individuals from the main subcohort, who were live-born during January 1st 1989- December 31st 1999 in Denmark, and had DBS cards with sufficient material for analysis and available vitamin D results (n=1600). Also, a random sample 1,039 fracture cases from a total of 82,154 fracture cases, who had been admitted to the hospital with a fracture of the forearm, wrist or scaphoid bone (ICD-10: S52, S62.0); the clavicle (ICD-10: S42.0); or the ankle (ICD-10: S82.5, S82.6, S82.8) was selected, as these sites are the most common among European children (5). For the present study, information on cause of fracture has not been assessed from the NPR. The NPR includes diagnosis codes and procedure codes for all treatments and also contains outpatient and emergency room contacts (26). The fractures occurred when the children were 6-13 years. If the child had more than one fracture admission, we only counted the first admission. There were 4 fracture cases which were also part of the random subcohort, which we attributed to the random subcohort. Flow chart of the study population is presented in **Figure 1.**

The Danish registers used in this study have coverage of almost the entire population and good validity of fracture diagnose recordings in NPR based on adult fracture patients with primary hyperparathyroidism (27). The accuracy is expected to be similar in children, since treatment of paediatric and adult fractures takes place at the same hospital units.

ETHICAL CONSIDERATIONS

Permission to access and analyze the DBS samples from the Biological Specimen Bank for Neonatal Screening has been given from the Danish National Committee on Biomedical Research Ethics (J. no.: H-3-2011-126) and from the steering committee from the biobank. Permission from the Danish Data Protection Agency has also been granted (J. no.: 2012-41-116). Anonymous register-based studies are not required ethical approval according to Danish law.

## ASSESMENT OF VITAMIN D STATUS

Since May 1st, 1981, neonatal DBS samples taken by heel prick up to a week after birth have been collected for all newborns in Denmark. After routine screening for congenital disorders, residual DBS filter cards are stored at -20°C in a locked freezer at the Biological Specimen Bank for Neonatal Screening at the State Serum Institute (28). Previous studies have shown that storage times of 25(OH)D3 for more than 20 years do not seem to bias inter-individual variation in concentrations for a given birth cohort regardless of storage temperature and light exposure (29).

From the stored surplus card samples, one 3.2 mm punch, taken half-way from the center of the blood spot was obtained. This punch was used to measure the main circulating form of vitamin D, neonatal 25(OH)D3, as well as 25(OH)D2 (30), but not the C-3 epimer of 25(OH)D3.

The assay used was a highly sensitive liquid chromatography tandem mass spectroscopy method (LC-MS/MS) run at the State Serum Institute using a modified version of Eyels and colleagues’ method (29). There was acceptable precision for all measured concentration levels for intra-assay and inter-assay analyses. The variability coefficient (CV%) for intra-asssay and inter-assay variation for 25(OH)D3 ranged 7-12% and 7-20%, respectively. Laboratory investigators were blinded to the diagnosis as well as the season of birth.

Since capillary blood during the neonatal period has significantly higher haematocrit (Hct) values compared to venous blood (31), we adjusted the concentrations of 25(OH)D3 in DBS to equivalent serum concentrations, using the following formula: serum (25(OH)D3) nmol/L = DBS (25(OH)D3) nmol/L \*1/[1-0.61 (the Hct fraction)] for capillary blood for newborns (31). We omitted all the 25(OH)D2 measurements, as only 4.5% were above the detection limit of 3 nmol/L. The concentration of 25(OH)D3 is reported in nmol/L.

ASSESSMENTS OF COVARIATES

Information on some maternal characteristics (educational level, ethnicity) was obtained from Denmark Statistics and smoking during pregnancy, age, parity, and gestational age was obtained from the Danish National Birth Register. *Educational level* was recoded into three levels: 1) Basic school 8th -10th class 2) General upper –secondary education, short-cycle higher education or vocational education and training 3) Medium or long-cycle higher education or bachelor. *Ethnicity* was defined as maternal European and Non-European origin. Hence, children with fathers with Non-European origin were considered European if mothers were of Western origin. There was only information on maternal *smoking* during pregnancy for a subsample of a total of 1982 individuals, of which 798 individuals were from the case group, since information on smoking during pregnancy only is available from Danish registries for individuals giving birth from 1991 and onward, where the collection of the smoking information started. Since 1997, the data on smoking during pregnancy was collected electronically, thus the information collected during 1991 to 1996 may not be complete. The smoking variable from 1991 to 1996 was grouped into smoking and non-smoking during pregnancy, and from 1997 and onward the cigarette smoking was reported in the following groups: unknown, mother not smoking, mother smokes, mother stopped smoking in 1st trimester, mother stopped smoking after 1st trimester, mother smokes up to either 5, 6 to 10, 11 to 20 or more than 20 cigarettes per day, respectively. When combining these information sources, we categorized the smoking variable into unknown, smoking, non-smoking during pregnancy. Mothers were considered smokers if they had ever smoked during pregnancy. The reporting of *parity* changed during the study period. From 1973-1996 the register included information on live and still births, only. During this period, we used the sum of births plus the actual births to estimate parity. Subsequently, parity was dichotomized into primiparous and multiparous. Maternal and gestational age were included in the analyses as continuous variables.

STATISTICAL ANALYSIS

In the descriptive data, the cases and the individuals from the random subcohort were compared using χ2-test for categorical variables and two sample t-test for continuous variables.

Unlike the classic case-cohort design (32) only a random sample of the fracture cases were included, and since loss to follow-up was rare among the individuals in the subcohort (n= 9) the data was analyzed as a case-control sample by only including individuals with complete follow-up.

Using logistic regression, we estimated unadjusted and adjusted ORs and 95% CI for the risk of fractures of the forearm, wrist, scaphoid bone, clavicle or ankles, whichever came first, in the ages 6-13 years in relation to 25(OH)D3 as a continuous variable, or as a categorical variable (quintiles of the distribution of 25(OH)D3 in the subcohort) in order to capture a potential non-linear relationship. Based on our hypothesis, that neonatal serum 25(OH)D3 concentrations is lower among children who go on to sustain fractures in childhood, the lowest category was chosen as reference. A priori, the model was adjusted for offspring, sex, gestational age, parity and maternal educational level, as these factors have previously been associated with neonatal bone mineralization (33). Moreover we adjusted for maternal age, since maternal age seems to be associated with both vitamin D status during pregnancy as well as offspring fracture risk during childhood (34;35). Based on theoretical plausibility presented in the background, potential interactions between the vitamin D quintile categories and maternal ethnicity were examined by adding interaction terms to the model and testing the statistical significance.

Since only a limited number of individuals had information on smoking (n=1982), the likelihood-ratio test was used to estimate whether adjusting for smoking in the model would improve the fit of the logistic model. Also, the individuals with missing information on maternal smoking during pregnancy were compared to the individuals with information on maternal smoking, to address selection bias using χ2-test and two sample t-test. The 25(OH)D3 was square root transformed to achieve normal distribution before performing the two sample t-test. To further explore the role of maternal smoking during pregnancy, sensitivity analysis were performed on the subset with available information on smoking during pregnancy, to test if maternal smoking was associated with fracture risk in the offspring using logistic regression, as well as examining the distribution of maternal smoking during pregnancy in relation to the quintiles of the distribution of 25(OH)D3 using χ2-test. Also, we tested whether there was effect modification with maternal smoking during pregnancy, by adding interaction terms to the model and testing the statistical significance.

As the 25(OH)D concentrations change according to the seasonal variation in sun exposure at northern latitude in both pregnant women and their offspring at birth (36), we performed pre-specified sensitivity analysis adjusting for season of birth, included season of birth as an interaction term in the further analysis. The season was defined as November to January, February to April, May to July and August to October based on the seasonal variation in serum 25(OH)D concentrations among individuals from countries from northern latitude (36;37). A likelihood-ratio test was used to estimate whether including ethnicity or season of birth in the model would improve the fit of the logistic model.

Sensitivity analyses were performed to test whether there was effect modification with sex, by adding interaction terms to the model and testing the statistical significance. A likelihood-ratio test was used to estimate whether including sex in the model would improve the fit of the logistic model.

Finally, global tests were performed as sensitivity analysis, to examine if there were any overall associations.

Statistical analyses were carried out in Stata version 14.1. A p-value of < 0.05 was considered significant.

**Results**

In the entire sample, the distribution of 25(OH)D3 was skewed. The mean (SD) for all subjects was 27.7 (18.9) nmol/L (median, 23.5; IQR, 13.3, 37.3), when corrected for the Hct fraction in capillary blood. As expected, 25(OH)D3 showed statistically significant monthly variation (p<0.001) with highest values in July and August and the lowest value in April among the individuals from fracture cases and subcohort combined (**Figure 2**).

In **Table 1** background characteristics are presented for the 1,039 fracture cases and the 1,600 children from the subcohort. There was no significant differences between the fracture cases and the controls in regards to sex, gestational age, birthweight, 25(OH)D3 quintile categories, parity or maternal educational level. There were borderline more maternal smokers among the case group compared to the subcohort (24 % versus 20 %, p=0.05) and the individuals with missing information on maternal smoking were primarily born in 1989 and 1990.

Also, there was a lower percentage of individuals with maternal non-European ethnic origin in the case group compared to the subcohort (4.3 % in the case group and 7.7 % in the subcohort (p<0.001)).

The odds for sustaining fractures were lower in the mid (third) quintile of 25(OH)D3 in both the crude (OR3: 0.76; 0.60, 0.98), the adjusted model 2 (OR3: 0.75; 95% CI: 0.58, 0.96) (**Table 2**), when compared to the first quintile. Further adjustment for smoking, season of birth, as well as birthweight, showed a similar pattern as model 2 (**Table 2**). There was no association between 25(OH)D3 as a continuous variable and fracture risk (data not shown). However, the results from the global tests did not show any significant overall associations in any of the models (**Table 2**).

Maternal smoking during pregnancy as exposure did not predict odds of fractures (OR: 1.19, 95% CI: 0.98, 1.45), but did seem to influence the 25(OH)D3 concentrations, both when using 25(OH)D3 concentrations as a continuous variable (p<0.0001) and as quintiles of 25(OH)D3 (p<0.001).

There was no significant interaction between vitamin D concentration categories and either ethnicity, sex, smoking, or season of birth in relation to odds of fractures (S**upplementa**l **Figure1-4**).

**Discussion**

In this large population-based randomly selected group of children born in Denmark during 1989-99, we found no evidence for an association between neonatal 25(OH)D3 and odds for sustaining fractures in childhood at age 6-13 years. Moreover, we found no interactions with ethnicity, sex, smoking or season of birth. In the analysis with maternal smoking during pregnancy as exposure, the 25(OH)D3 concentrations were lower, but were not associated with odds of fracture.

Our findings agree with results from the recent Danish Fetal Origins 1988 Cohort study (n=850) (9), and the Danish National Birth Cohort study (n=1497) (10), showing no association between antenatal 25(OH)D concentration and risk of fractures among the offspring during childhood. Given that the fetus relies entirely on the maternal status (38) and that low bone mineral density is predictive of increased fracture risk (6;39-45), our results are equivalent to the large observational ALSPAC study (14), and the randomized controlled MAVIDOS study (46), but in contrast to other observational studies which found that prenatal vitamin D status/UVB-exposure was associated with skeletal strength and development (11-13).

In the interpretation of the differences in the above mentioned studies, one may consider the etiology of childhood fractures (47); while the measurements of bone mass possibly capture the underlying skeletal deficits unrelated to the occurrence of an accident, the fracture events may characterize the degree of trauma, which was not distinguished in any of the fracture studies, and unfortunately, the cause of fracture was not retrieved from the Danish National Patient Register for the present study either, but may be of interest for future studies.

Comparable to previous studies (48-51), our sensitivity analyses suggested that in utero exposure to smoking influenced vitamin D concentrations, but not fracture risk. These results make us questioning whether or not we should adjust for maternal smoking during pregnancy from a confounding perspective. Nevertheless, the inclusion of smoking in model 3a did not improve the fit of the logistic regression, and the pattern of the estimates in this analysis was essentially similar to those from the other models.

The numerical values of 25(OH)D3 in the present study were low (mean 25(OH)D3: 27.4 (18.5) nmol/L), compared to most other studies using DBS (varied from 28.2-48.5 nmol/L) (16;17;19;21;23), but importantly, also compared to studies measuring 25(OH)D3 ina similar subsample of Danish neonates (McGrath et al.: 35.9 (21.0) nmol/L and Nielsen et al.: Cases: 33.0 (16.9) nmol/L / Controls: 35.9 (17.5) nmol/L) (15;20). A number of potential explanations for the low values may be considered in the interpretation of our findings. Inter- variability between laboratory both within and between countries are to be expected (52), especially since there currently are no quality assurance program for vitamin D in dried blood spots. However, our laboratory participates in the Vitamin D External Quality Assessment Scheme program with the equivalent serum method. Especially dissimilarities in the separation methods may be considered, as the C3-epiform of 25(OH)D3 was not included in our measurements. This is relevant, since the LC-MS/MS assays has shown elevated concentrations of 25(OH)D3 in infants due to the presence of the C-3 epimer of 25(OH)D3 (53). Additional explanations may be related to

deviation from detailed guidelines of the blood collection procedure, where the most common errors are overfilling, partial fillings or multiple applications on the circles on the filter paper (54); punch position, because findings have revealed that 25(OH)D concentrations differs across the spots, with highest concentrations in the periphery of the spots and lowest in the center (54); and/or multiple freeze-thawing cycles, as a result of the stored DBS samples from the 1980ties were subjected to many punches in relation to analysis in other previous research projects. Multiple freeze-thaw cycles in serum 25(OH)D seem to be valid (55), and we assume that the validity is also applicable to 25(OH)D measurements in DBS samples, although this has not been formally tested.

However, we have no reason to believe that the generally low values influenced the ranking of subjects, and hence no reason to question the validity of our associations. Indeed, the seasonal variation in the 25(OH)D3 concentrations was well captured in the present study for both the cases and the subcohort.

STRENGTHS AND LIMITATION OF THE STUDY

There are some strengths and limitations to the study which needs to be taken into account in the interpretation of the findings (56). The strengths of the present study lies in the large sample size of individuals randomly selected from the entire Danish population and coupled with neonatal biomaterial (n=2639). All but one (14) of previously published studies that we are aware of, included less than 1500 mother-offspring pairs.Also, apprehensions regarding selection bias are negligible, since information on the mother-offspring pairs in the present analyses were derived from national registries, and the obtainment of the Danish neonatal DBS samples are close to complete. Even though we were able to adjust for several potential confounders through the use of information from comprehensive registers with high validity and covering the entire Danish population, there may still be a risk of residual confounding from variables that we lacked information on, although adjusting for sociodemographic factors such as educational level and maternal age, as well as behavioral factors such as parity and maternal smoking, may be considered as proxy variables for other life-style factors in general, including supplement use and physical activity level (56;57).

IMPLICATION FOR POLICY MAKERS

Policy making strategies in relation to primary prevention of fractures among children cannot be derived from the results at this point, but ongoing randomized controlled trials with supplementation of vitamin D in pregnancy (46;58;59), awaits the first bone fracture outcome data in the children, in order to properly inform health policy.

**Conclusion and future studies**

Overall, neonatal vitamin D status does not seem to influence the subsequent fracture risk in childhood. These results are in line with results from the few previous studies that examined associations between antenatal maternal vitamin D status and childhood fractures. Large randomized controlled trials with vitamin D supplementation during pregnancy in relation to fracture outcome are needed at this point.

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**Author Contribution:** MNH, BA and BLH initiated the study, participated in its design and coordination. MNH and PF performed the statistical analysis. AC performed the 25(OH)D measurements on the DBS. MNH, BA and BLH wrote the paper with contributions from all authors. MNH has primary responsibility for the final content. All authors have read and approved the final manuscript. MNH affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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**Table 1**. Maternal and offspring background characteristics for individuals from the case group and the random subcohort

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Fracture cases | | Random subcohort | |  |
|  | n | Mean (SD) or % | n | Mean (SD) or % | p |
| OFFSPRING |  |  |  |  |  |
| Fracture event of forearm, wrist, scaphoid bone, clavicle or ankle at age 6-13 years, % | 1039 | 100 | 1565 | 12.9 | **<0.001** |
| Sex, girls, % | 1039 | 50.1 | 1565 | 47.7 | 0.23 |
| Gestational age, week | 1023 | 39.5 (1.8) | 1537 | 39.6 (2.0) | 0.28 |
| Birth weight, g | 1029 | 3494 (561) | 1538 | 3497 (578) | 0.87 |
| Quintiles (mean 25(OH)D3; nmol/L) | 1039 |  | 1565 |  | 0.29 |
| Q1 (7.88), % |  | 22.1 |  | 19.9 |  |
| Q2 (15.33), % |  | 21.1 |  | 19.9 |  |
| Q3 (23.90), % |  | 17.1 |  | 19.9 |  |
| Q4 (33.93), % |  | 20.7 |  | 20.2 |  |
| Q5 (53.18), % |  | 19.0 |  | 20.1 |  |
| MATERNAL |  |  |  |  |  |
| *Smoking* | 1039 |  | 1565 |  | **0.05** |
| smoking during pregnancy, yes, % | 248 | 23.9 | 311 | 19.9 |  |
| smoking during pregnancy, no, % | 550 | 52.9 | 873 | 55.8 |  |
| Maternal age | 1036 | 28.2 (4.6) | 1549 | 28.5 (4.7) | 0.13 |
| Multiparous, yes, % | 1036 | 54.5 | 1548 | 55.7 | 0.57 |
| *Educational level* | 1014 |  | 1513 |  | 0.46 |
| Basic school 8th -10th class, % |  | 29.6 |  | 27.3 |  |
| General upper –secondary education, short-cycle higher education or vocational education and training, % |  | 49.7 |  | 51.4 |  |
| Medium or long-cycle higher education or bachelor, % |  | 20.7 |  | 21.4 |  |
| *Immigrant background* | 1039 |  | 1560 |  | **0.001** |
| European, % |  | 95.7 |  | 92.3 |  |
| Non-European, % |  | 4.3 |  | 7.7 |  |
| The cases and the individuals from the subcohort were compared using χ2-test for categorical variables and two sample t-test for continuous variables. | | | | | |

**Table 2**. Odds Ratio (OR) and 95% confidence intervals (CI) of fractures in offspring at age 6-13 years to the concentration of 25(OH)D3, by the quintiles of the distribution of 25(OH)D3.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Odds Ratio (95% Confidence Interval) | | | | |
|  | Model 1 | Model 2 | Model 3a | Model 3b | Model 3c |
|  | n=2604 | n=2559 | n=2559 | n=2559 | n=2547 |
| Quintiles  (mean 25(OH)D3; nmol/L) |  |  |  |  |  |
| Quinti1e 1  (7.88) | 1 (ref) | 1 (ref) | 1 (ref) | 1 (ref) | 1 (ref) |
| Quinti1e 2 (15.33) | 1.03 (0.81, 1.31) | 1.00 (0.78, 1.28) | 1.01 (0.79, 1.30) | 0.98 (0.76, 1.25) | 0.99 (0.77, 1.26) |
| Quinti1e 3 (23.90) | **0.76 (0.60, 0.98)** | **0.75 (0.58, 0.96)** | **0.76 (0.59, 0.98)** | **0.72 (0.55, 0.93)** | **0.74 (0.57, 0.95)** |
| Quinti1e 4 (33.93) | 0.98 (0.77, 1.24) | 0.94 (0.73, 1.20) | 0.96 (0.74, 1.23) | 0.88 (0.68, 1.14) | 0.92 (0.72, 1.18) |
| Quinti1e 5 (53.18) | 0.92 (0.72, 1.17) | 0.89 (0.69, 1.15) | 0.91 (0.70, 1.17) | 0.81 (0.62, 1.07) | 0.88 (0.68, 1.14) |
| Global test,  p-value | 0.13 | 0.13 | 0.16 | 0.08 | 0.14 |
| Model 1: Logistic regression.  Model 2: Model 1 combined with adjustment for sex, gestational age, parity, maternal educational level, maternal ethnicity, and maternal age.  Model 3a: Model 2 combined with adjustment for maternal smoking during pregnancy. Likelihood-ratio test: p=0.21.  Model 3b: Model 2 combined with adjustment for season of birth. Likelihood-ratio test: p=0.43.  Model 3c: Model 2 combined with adjustment for birthweight. Likelihood-ratio test: p=0.07. | | | | | |

**Figure 1.** Flow chart of the study population. 1 Dried blood spots-cards not found, insufficient material for analysis or analysis failed.

**Figure 2**. Box plot of the median 25(OH)D3 concentrations (nmol/L) by month of birth of the individuals from the total study population (n=2604). The boxes indicate the interquartile range, the vertical lines indicate the range, and the dots indicate outliers. The dried blood spot 25(OH)D3 concentrations have been corrected for the hematocrit fraction of 61% for capillary blood. Test for no interaction between month of birth and sampling status (cases versus subcohort) (p=0.48). Test for no difference in 25(OH)D3 concentrations between cases and subcohort adjusted for month of birth (p=0.54).