Web Material

Supplementary information for Thorsen *et al*. - Maternal and Neonatal Vitamin D Status are not associated with Risk of Childhood Type 1 Diabetes: a Scandinavian Case-Cohort Study

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Web Appendix 1

Assessment of vitamin D status

All calibrators, controls, internal standards, reagents, and microtiter plates were from the MSMS Vitamin D kit (PerkinElmer, Inc., Waltham, MA, USA) for mass spectrometry (MS). Acetonitrile (ACN) was purchased from Sigma-Aldrich (St. Louis, MO, USA), methanol from Riedel-de Haën (Hanover, Germany), and formic acid from Merck (Darmstadt, Germany). Water was purified using a water purification unit (Merck Millipore, Billerica, MA, USA).

The liquid chromatography-mass spectrometry (LC-MS) system consisted of high performance liquid chromatography with a Surveyor MS Pump Plus (Thermo Fisher Scientific, Waltham, MA, USA) and an HTS PAL sample handling system (CTC Analytics AG, Zwingen, Switzerland) connected to a TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific) equipped with Electrospray Ionisation (ESI). Analytical separation was achieved using a Hypersil Gold reversed phase column (50 × 2.1 mm, 3 µm) and a Hypersil Gold guard column (10 × 2.1 mm, 3 µm) (Thermo Fisher Scientific). Solvent A consisted of a mixture of Milli-Q water and methanol (1:1) containing 250 µL/L of additive and Solvent B consisted of 100% methanol containing 250 µL/L of additive. The exact composition of the additive is undisclosed but includes methylamine. Prior to MS, analytes were separated by gradient elution using 50–100% of Solvent B from 0–1.6 min at a flow rate of 0.4 mL/min.

The MS system was operated in positive mode using ESI and the following source settings: spray voltage, 3,000; sheet gas, 45; ion sweep gas, 5.0; aux gas, 40; cap temp, 300; and skimmer offset, –7. Collision voltages and T-lens voltages were optimised for each transition, and derivatized analytes were detected as methylamine adducts. The following transitions were used: 25-hydroxyvitamin D_2 ($25(OH)D_2$) [619.4/298.1] and 25-hydroxyvitamin D_3 ($25(OH)D_3$) [607.3/298.1]. For the internal standards the following transitions were used: $^2H_3$-25-hydroxyvitamin D_2 [622.3/301.1] and $^2H_3$-25-hydroxyvitamin D_3 [610.3/301.1]. For the calibrators $^2H_6$-25-hydroxyvitamin D_2 [625.4/268.1] and $^2H_6$-25-hydroxyvitamin D_3 [613.3/298.1] were used.

The MSMS Vitamin D kit calibrators and controls were reconstituted in water according to the manufacturer's instructions (PerkinElmer, Inc.). A deproteinising solution (DPS) consisting of ACN and 0.1% formic acid containing internal standard was prepared. Aliquots (5 µL) from each calibrator, control, and serum sample was pipetted onto a 96-well microtiter plate. A total of 120 µL of DPS was added to precipitate proteins from the samples. The plate was sealed with a silicone plate mat and shaken for 10 min at room temperature before being centrifuged at 4,000 rpm for 30 min at 4°C. Supernatants were transferred to a new microtiter plate and dried under a stream of nitrogen gas. A total of 50 µL of derivatization reagent [containing PTAD (4-phenyl-1,2,4-triazoline-3,5-dione)] dissolved in ethylacetate was added to each well and the plate was shaken for 10 min before reactions were quenched with 25 µL of 99.9% ethanol. The plate was subsequently dried under a stream of nitrogen gas. The extracts were reconstituted by adding 80 µL of a mixture of Milli-Q water and ACN (1:1) to each well and the plate was shaken for 10 min at 500 rpm. The plate was then covered with aluminium sealing foil and centrifuged at 3,000 rpm for 10 min at 4°C prior LC-MS analysis. A total of 20 µL of each sample was injected into the LC-MS system.

In total, 95.6% of the Danish National Birth Cohort (DNBC) and 99.6% of the Norwegian Mother and Child Cohort (MoBa) samples had concentrations of $25(OH)D_2$ that were below the lower limit of quantification (LLOQ; 5.8 nmol/L) and these were set to zero in the statistical analysis. In addition, 2% of all samples (DNBC and MoBA) had concentrations of $25(OH)D_3$ that were below the LLOQ (8.5 nmol/L) and these were set to 50% of LLOQ in the statistical analysis.

Our laboratory, at the Statens Serum Institut, participates in the vitamin D external quality assessment scheme (DEQAS).

Questionnaires
The questionnaires used for obtaining covariate information in the Danish National Birth Cohort and The Norwegian Mother and Child Cohort Study is accessible at the respective study websites (1, 2).

Statistical analysis

Pre-study power analysis: The Norwegian (MoBa) and Danish (DNBC) sub-studies were each shown in our pre-study calculations to have 91% statistical power to detect a true association of magnitude similar to that observed in a previous study by Sørensen et al. (3). By combining the two sub-studies and thus doubling the sample size, we should have ample power to detect a clinically relevant association.

Our primary analysis tests our hypothesis that estimated average 25(OH)D concentrations during pregnancy are predictive of childhood T1D. The rationale for this is twofold: 1) Although there were some evidence for a stronger association in the third trimester than earlier in pregnancy in a previous study (4), this previous study did not provide strong evidence for a statistical interaction between gestational age and 25(OH)D. In other words there were no strong a priori evidence for a trimester-specific association. 2) The 25(OH)D concentrations are subject to considerable intra-individual variation, which if ignored can lead to an underestimation of the regression parameters, known as attenuation bias (5). To alleviate this problem and thus increase the statistical power, we applied a measurement error (structural equation) model with an estimated average 25(OH)D concentration, D_i, for each subject $i = 1, \ldots, n$:

$$Y_{ij} = a_j + \lambda_j D_i + X_{ij}^t \beta_j + \epsilon_{ij},$$

where Y_{ij} is the jth measurement (1: first trimester; 2: second trimester; 3: post-partum; and 4: umbilical cord vein blood) in the ith mother/child pair, X_{ij} is observed covariates, and ϵ_{ij} is a measurement error term assumed to follow a zero-mean Gaussian distribution independently of the other residual terms, the covariates, and the estimated average 25(OH)D concentration D_i. The covariates, $X_{ij,}$ we adjusted for were seasonal differences (cosine and sine transformations with a period of 1 year from the day of blood sampling) and gestation age (linear) at sample collection. For each individual we can then predict the latent 25(OH)D concentration during pregnancy using the conditional expectation (Best Linear Unbiased Predictor)

$$\widehat{D}_i = E(D_i | Y_i, X_i),$$

where Y_i and X_i are the vectors of the available observations for individual i. The measurement error model was estimated using the Maximum Likelihood method, allowing us to take missing 25(OH)D concentration measurements into account under a Missing At Random (MAR) assumption. Our analyses are otherwise based on complete cases, i.e., includes those with information on all covariates, although the proportion of subjects with missing covariate data was limited and the influence of the missing covariate information was assessed using sensitivity analyses (see below). The estimated average 25(OH)D concentrations were then used as predictors in the weighted Cox regression corresponding to a first order approximation of the observed hazard function and conditional on the estimated average 25(OH)D concentration and other covariates. This *calibration* method performs very well under different conditions, and with negligible bias under a rare event assumption (6), as in this study.

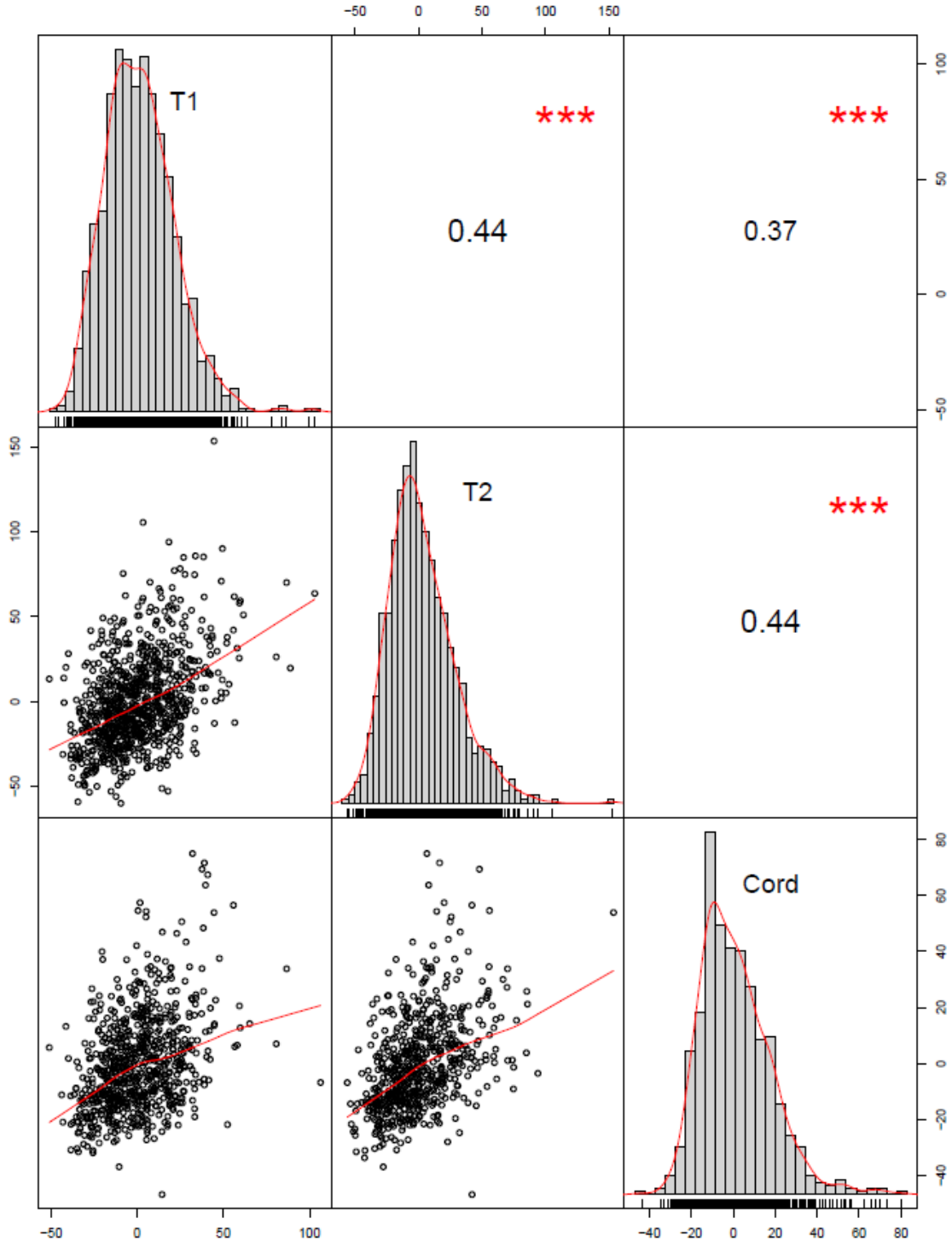
The measurement error model was used with the second trimester measurement as the reference indicator, i.e., with $\lambda_2 = 1$, and hence regression coefficients in the survival analysis, after exponential transformation, can be interpreted as hazard ratios for a 10 nmol/L increase in second trimester 25(OH)D concentrations. To account for the uncertainty of both the measurement error model and the survival analysis, the 95% confidence intervals (CI) for the primary analysis were calculated using non-parametric bootstrapping (5,000 resamples with the basic bootstrap CIs). Because the standard 95% confidence intervals from the Cox regression model gave essentially the same results, we used this for all analyses following the primary analysis.

To assess the potential for selection bias due to missing covariate data (birth weight, 4; maternal age, 1; and maternal pre-pregnancy BMI, 152) inverse probability weighting was applied using a logistic regression model with interaction between the mode of delivery and maternal age. The weights were combined using the sampling weights in the weighted Cox regression.

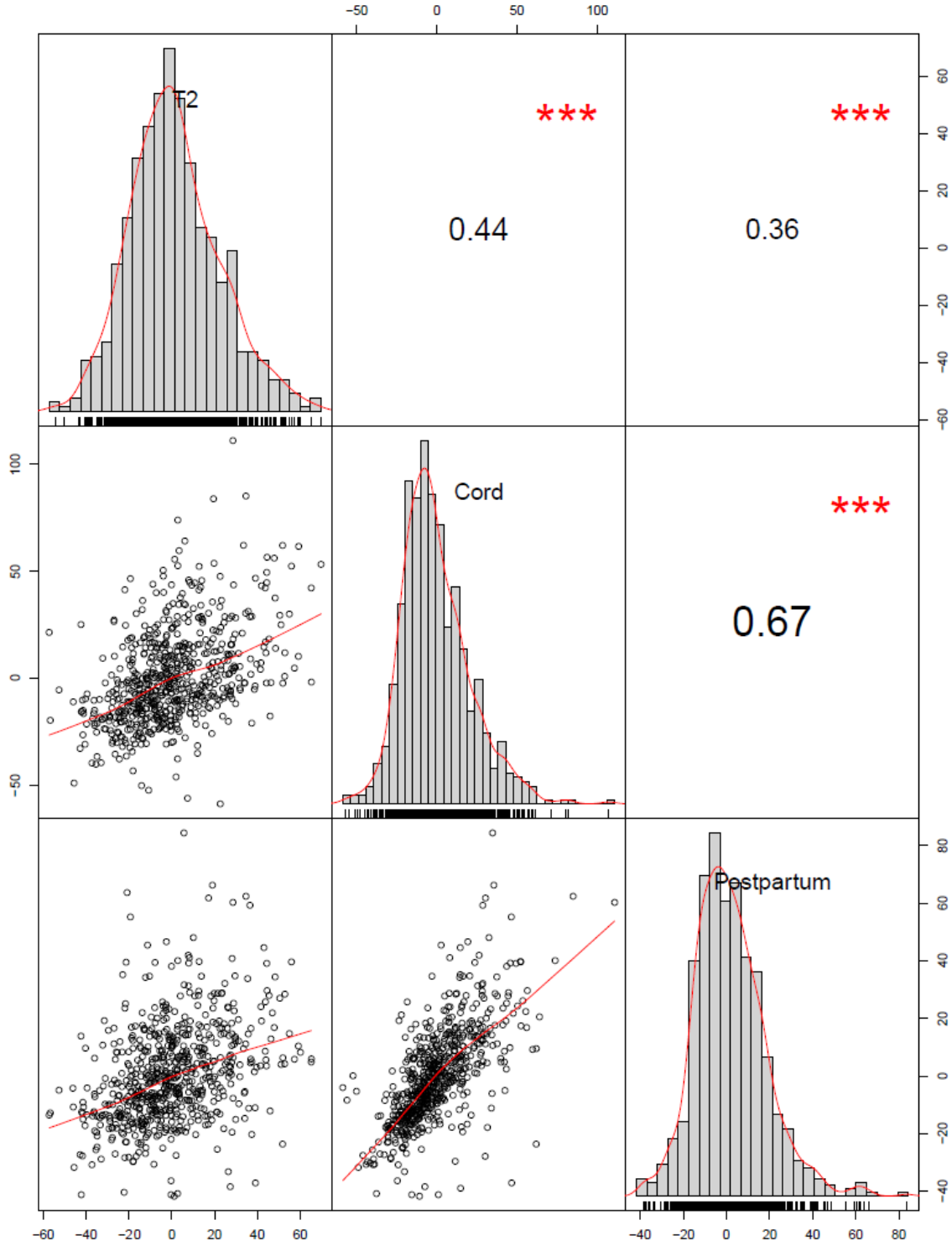
PerformanceAnalytics software (ver. 3.2.2) was used to graphically present cohort-specific correlations among seasonally-adjusted residuals of 25(OH)D concentrations in different sample types (Web Fig. 1a, b).

Web Appendix 2

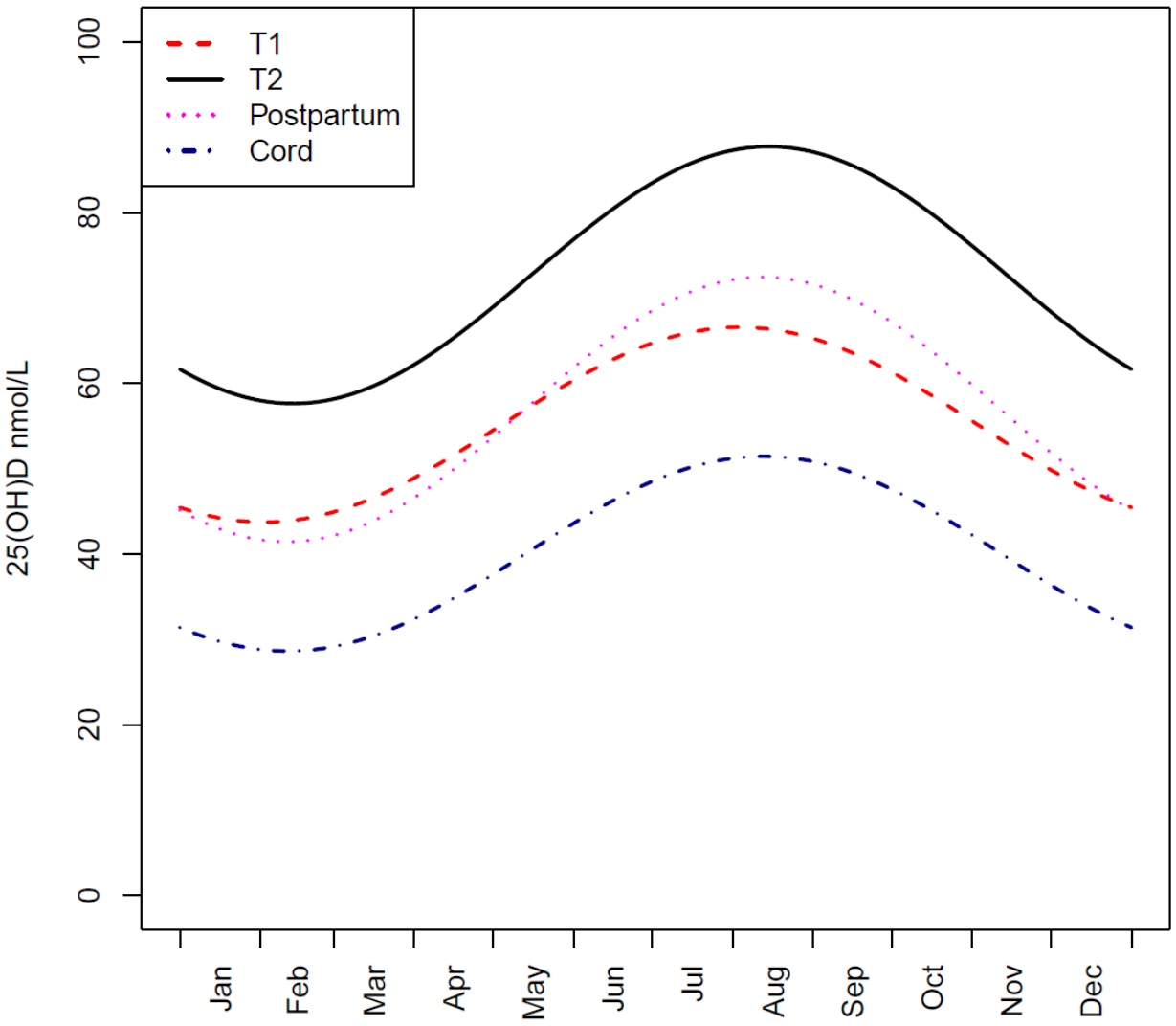
Web Figure 1a. Distribution of seasonally-adjusted residuals of 25-hydroxyvitamin D concentrations in the three sample types from the Danish National Birth Cohort (diagonal panels); scatterplots for pairs of sample types (lower left panels); and Pearson correlation coefficients for pairs of sample types (upper right panels). Units marked on the outside are in nmol/L, and zero corresponds to the mean value (residuals are deviations from the mean value). T1: first trimester samples (gestation week 7–9); T2: second trimester samples (gestation week 24–25); and Cord: umbilical cord samples. *** P < 0.001.



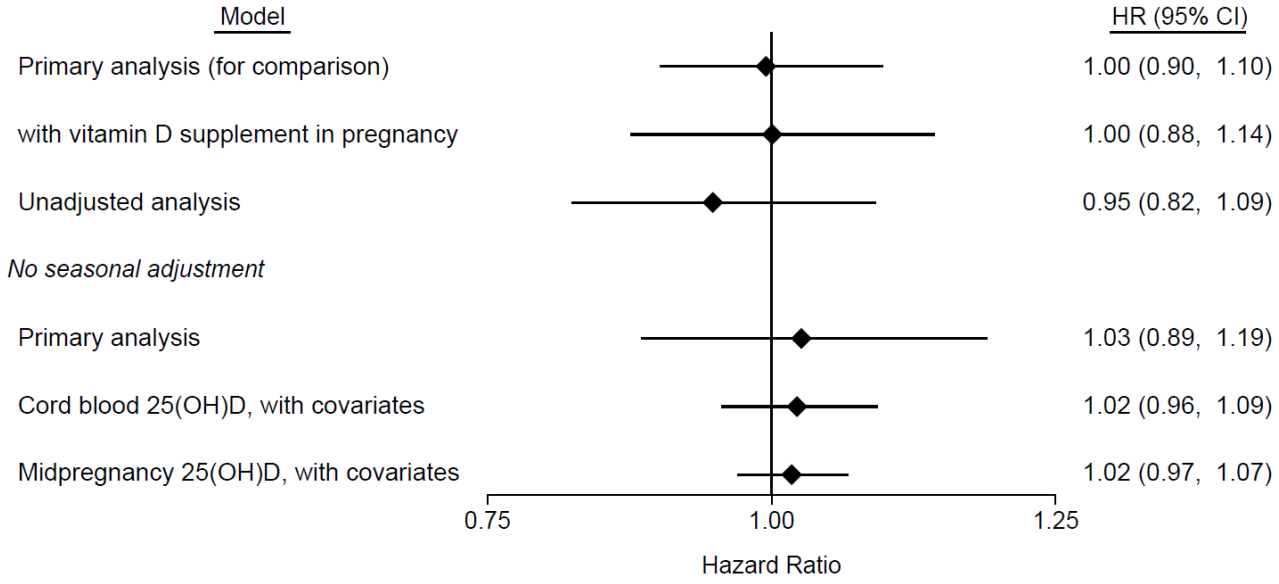
Web Figure 1b. Distribution of seasonally-adjusted residuals of 25-hydroxyvitamin D concentrations in the three sample types from the Norwegian Mother and Child Cohort Study (diagonal panels); scatterplots for pairs of sample types (lower left panels); and Pearson correlation coefficients for pairs of sample types (upper right panels). Units marked on the outside are in nmol/L, and zero corresponds to the mean value (residuals are deviations from the mean value). T2: second trimester samples (gestation week 17–18); Cord: umbilical cord samples; and Postpartum: median of two days after delivery. *** P < 0.001.



Web Figure 2. Seasonal variations in mean plasma 25-hydroxyvitamin D (25(OH)D) concentrations by sample type in the Norwegian Mother and Child, and Danish National Birth Cohorts. T1: first trimester samples (gestation week 7–9, Danish cohort only); T2: second trimester samples (gestation week 17–25); Postpartum: maternal samples taken within a median of two days after birth (Norwegian cohort only); and Cord: umbilical cord plasma.



Web Figure 3. Additional sensitivity analyses for the association between maternal and umbilical cord blood vitamin D status and the risk of childhood type 1 diabetes (hazard ratio per 10 nmol/L increase in plasma 25(OH)D concentration). The primary model was adjusted for maternal diabetes, age at time of delivery, pre-pregnancy BMI, child's sex, birth weight, and the time of year (season) when the blood sample was taken.



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