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The Role of Vitamin D on Circulating Memory T Cells in Children: The Generation R Study

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ABSTRACT PAGE

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Background Previous studies have demonstrated that vitamin D affects T cell function and maturation via the vitamin D receptor. However, no studies in children have been performed on this topic. Because most of the T cell memory is formed in the first five years of life, we aimed to determine the association between serum 25-hydroxyvitamin D (25(OH)D) levels and numbers of circulatory naive, central memory (T_{cm}) and effector memory (T_{em}) T lymphocytes in a large population of healthy children.

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Methods Among 3,189 children participating in a population-based prospective cohort, we measured 25(OH)D levels and performed detailed immuno-phenotyping of naive and memory T-lymphocytes at a median age of 6.0 years (95% range 5.7 to 7.9). Detailed lymphocyte subsets were available in 986 children. Multivariable linear regression analyses were performed to determine the association between 25(OH)D and the maturation of T lymphocytes in children adjusted for cord blood 25(OH)D levels, herpes seropositivity, sociodemographic and lifestyle confounders. Furthermore, multivariable logistic regression analyses were performed to determine associations between 25(OH)D and childhood infections.

Results Higher 25(OH)D levels were associated with higher numbers of Tem lymphocytes. Every 10 nmol/L higher 25(OH)D was associated with 2.20% (95% CI 0.54-3.89; $p=0.009$) higher CD4TemRA, 1.50% (95% CI 0.38-2.62; $p=0.008$) higher CD4TemRO and 1.82% (95% CI 0.11-3.56; $p=0.037$) higher CD8TemRA cell numbers. Generally, stronger associations were observed among boys. 25(OH)D levels were not significantly associated with naive, Tcm cell numbers, herpes seropositivity or URTIs.

Conclusions Our results suggest that vitamin D enhances cellular immunity in young children.

Key words: Adaptive Immune System, Calcitriol, Child, T lymphocytes, Vitamin D.

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INTRODUCTION

The active metabolite of vitamin D, also known as calcitriol, has preventive effects on several diseases, including bone diseases, cancers, infections and autoimmune diseases.(1)

Due to its effect on the control of infections and development of autoimmune diseases, vitamin D is thought to affect T lymphocytes: the CD4+helper T cells and the CD8+cytotoxic T cells.(2) CD4+T cells regulate both the activation of CD8+T cells and the maturation of B cells into memory B cells and Ig-producing plasma cells, whereas CD8+T cells induce cell death of pathogen-infected or malignant cells.(2) Memory T cells can be classified into central memory cells, circulating in blood and lymphoid tissues, and effector memory cells, which migrate to affected tissues.(3) Within effector memory T cells (Tem), a further

distinction can be made based on the expression of either the CD45RO or the CD45RA splice variants, where the latter ones are more differentiated.(3)

Vitamin D may play a role in the formation of memory T cells, because activated T cells express the vitamin D receptor (VDR).(4, 5) In cooperation with chromatin modification enzymes as histone acetyltransferases and histone deacetylases, the VDR is important in the regulation of genes that regulate cellular differentiation, apoptosis and proliferation of (non-)cancer cells.(4, 6, 7) VDR knock-out mouse models showed increased proliferation of T cells with limited differentiation.(8) This is in line with observations from adult studies.(9, 10) The effects of vitamin D on T cell activation are likely to affect the formation of immunological memory.(9, 10)

No studies on 25-hydroxyvitamin D (25(OH)D) and the maturation of T memory cells in children have been performed. Because T cell numbers are highly dynamic in the first five years of life after which these stabilize, we examined the association between 25(OH)D and circulatory naive, central memory and effector memory T lymphocyte numbers in 3,189 six-year-old children.

METHODS

Study Design

This study was embedded in the Generation R Study, a population-based prospective cohort in the Netherlands, and has been described elsewhere.(11) For all children written informed consent was obtained. The Medical Ethical Committee of Erasmus Medical Center approved the Generation R Study.(11) Peripheral non-fasting blood samples were obtained by antecubital venipuncture to determine serum 25(OH)D and blood T-cell subsets at six

years.(12) We included six-year-old children with available measurements of serum 25(OH)D and T,B,NK cells(n=3,189). In this population, lymphocyte subsets were available in 986-990 children.

Assessment of vitamin D

Serum was extracted from peripheral blood samples and was stored at -80°C until quantification of 25(OH)D levels using isotope dilution on-line solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS).(12) The lower detection limit of 25(OH)D was 4.0 nmol/L. Intra-assay and inter-assay coefficients of variation of the 25(OH)D analyses for concentrations between 25-180 nmol/L were <6% and <8%, respectively.(12) Children were categorized into three groups based on vitamin D status: deficient (<50 nmol/L), sufficient (50 to <75 nmol/L), or optimal ($\geq$ 75 nmol/L).(12)

Assessment of basic blood lymphocyte subsets

Lymphocyte subsets were determined within 24 hours following blood sampling. Absolute numbers of CD3+T cells, CD19+B cells and CD16+/CD56+NK cells were obtained with a routine diagnostic lyse-no-wash protocol on a BD FACSCalibur (BD Biosciences).

Assessment of detailed blood lymphocyte subsets

Detailed analysis of T-cell subsets was performed with 6-color flow cytometry.(13, 14) T cells were categorized into CD4+ and CD8+ lineages, in which a further distinction was made between naive cells (CD45RO-CCR7+), central memory T cells (Tcm;CD45RO+CCR7+), effector memory RO positive T cells (TemRO;CD45RO+CCR7-) and effector memory RA positive cells (TemRA;CD45RO-CCR7-).

Covariates

Information on sex, birth weight and date of birth was obtained from midwife and hospital registries.(14) To take previous 25(OH)D levels into account, cord blood 25(OH)D levels were defined using ID-XLC-MS/MS. Information on siblings in household, ethnicity, and

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history of upper respiratory tract infections (URTI) at six years were obtained from medical records and questionnaires.(12) Sex-specific standard deviation scores (SDS) for body mass index (BMI, kg/m²) were calculated.(15) Seropositivity of CMV (IgG-CMV), herpes simplex virus 1 (IgG-HSV-1) and/or EBV (IgG-EBV-Viral capsid antigen(VCA)) were determined by ELISA from serum samples at six years.(16) Child ethnicity was determined as previously described and categorized into Western (Dutch, European, American, Oceanian) or non-Western (Turkish, Moroccan, African, Asian).(13, 15, 16) Child lifestyle characteristics as playing sports and time spent watching television, using a computer and/or playing outside at daytime were determined using questionnaires administered to the parents at the child age of six years.(12) Season of blood drawn was obtained from time of research center visit.(12) Household income, folic acid supplement use and smoking during pregnancy, were obtained with parental derived questionnaires.(14) Delivery reports and questionnaires derived at multiple consecutive time points provided information on maternal breastfeeding duration and exclusivity.(14)

Statistical Analyses

Kruskall Wallis rank sum tests, one-way ANOVA and chi-squared tests were conducted to determine differences in baseline characteristics between deficient, sufficient and optimal 25(OH)D levels. Natural log-scale transformations on lymphocyte numbers (cells per μ L blood) were performed. Multivariable linear regression analyses were performed to assess associations between 25(OH)D and immune cell numbers. Covariates were included in the final multivariable model if the covariate resulted in an alteration in effect estimate of at least 10%, for at least one of the outcomes.(17) Based on this criterion, we adjusted for the following child characteristics: sex, age, ethnicity, birth weight, number of children in household, IgG-CMV, IgG-HSV-1 and/or IgG-EBV-VCA, season of blood draw, BMI-SDS, playing sports, watching television and using a computer, playing outside at daytime, serum

25(OH)D at birth and URTIs at six years of age. Additionally, we adjusted for the parental characteristics household income, exclusive breastfeeding, start of folic acid use and maternal smoking during pregnancy. Effect modification by sex, ethnicity and age was evaluated by adding the interaction terms of each of these variables with 25(OH)D to our multivariable model. Binary logistic regression analyses were performed to determine associations between 25(OH)D and IgG-CMV, IgG-HSV-1 and/or IgG-EBV-VCA and a multinomial regression analysis was performed to determine the association between 25(OH)D and URTI numbers at six years old, with no infections as reference category. We adjusted for the same confounders as in the linear regression analyses. Because of strong correlations between our outcomes (Pearson's correlations >0.80), we did not perform adjustments for multiple testing. To reduce attrition bias, multiple imputation of the covariates was performed (n=5 imputations), using the Fully Conditional Specification method (predictive mean matching). The percentage of missing values in the original database ranged from 0.2%- 45%. Pooled regression coefficients of the five imputed datasets were reported in this paper as regression coefficients (β s) or odds ratios (ORs) with 95% confidence intervals (CI). Statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and R version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Study Population Characteristics

The study population consisted of 3,189 children (48.4% boys) with a median age of 6.0 years (95%CI 5.7 to 7.9). The median 25(OH)D level was 63.8 nmol/L (95%CI 17.0-131.0).

Children were classified into three groups based on the serum 25(OH)D levels: deficient (<50 nmol/L) in 31.5%, sufficient (50-75 nmol/L) in 33.6% and optimal ($\geq$ 75 nmol/L) in 34.9% (Table 1). Supplementary Table 1 shows the characteristics based on the original data.

Associations between 25(OH)D levels and T-cell numbers

To study the effects of 25(OH)D levels on blood lymphocytes, we performed multivariable linear regressions on each lineage as a whole (Table 2). Neither total T, B and NK cell numbers, nor naive CD4+ and CD8+ T cells were associated with differences in 25(OH)D levels. Subsequently, memory T cell subsets were analyzed. This showed positive associations between 25(OH)D levels and absolute numbers of CD4TemRO, CD4TemRA and CD8TemRA cells. A 10 nmol/L higher serum 25(OH)D was associated with 2.20% (95%CI 0.54 to 3.89, $p=0.009$) higher CD4TemRA numbers, 1.50% (95% CI 0.38 to 2.62, $p=0.008$) higher CD4TemRO numbers and 1.82% (95% CI 0.11 to 3.56, $p=0.037$) higher CD8TemRA numbers. No significant associations were observed between 25(OH)D and the Tcm subsets or between 25(OH)D and the CD8TemRO subsets. Figures 1 and 2 show the predicted natural log T cell numbers for 25(OH)D levels. Supplementary Table 2 shows the results stratified by 25(OH)D status.

Analyses stratified by sex

Because sex is likely to modify associations between vitamin D and lymphocyte numbers, we evaluated whether associations were different in boys and girls.⁽¹⁸⁾ Indeed, the interaction term between sex and 25(OH)D was significant ($p<0.030$) for multiple lymphocyte outcomes. Therefore, we stratified our analyses for sex. In general, stronger associations between 25(OH)D and blood lymphocytes were observed in boys than in girls (Table 3). Associations between 25(OH)D levels and CD4TemRA and CD8TemRA were stronger in boys, whereas the association between 25(OH)D levels and CD8TemRO was stronger in girls.

Associations between 25(OH)D levels and infections

To examine whether the observed associations between serum 25(OH)D and memory T cells could be explained by previous childhood infections, we determined the associations between 25(OH)D and IgG-CMV, IgG-HSV1 and IgG-EBV-VCA serpositivity and URTIs at 6 years

of age. However, 25(OH)D was not significantly associated with herpesvirus seropositivity nor number of URTIs after adjustment for confounding variables (Table 4).

DISCUSSION

In this large prospective cohort study, we observed that higher serum 25(OH)D levels were associated with higher memory T-cell numbers in six-year-old children. The associations were affected by sex: 25(OH)D had a pronounced positive effect on CD4TemRO numbers in girls, whereas in boys CD4TemRA and CD8TemRA were higher.

Previous Literature and Interpretation

This is the first study that determined associations between 25(OH)D and memory T cell numbers in a large population of healthy children. This study in children is relevant because early life exposures are crucial in the maturation of the immune system and disease susceptibility.⁽¹⁹⁾ Since T cell memory is long lasting, the conditions under which T cell memory is formed may have long-lasting effects on immunity.⁽¹⁹⁾

In line with our observations, previous studies reported positive associations between 25(OH)D and T lymphocyte differentiation into Tem cells, and between 25(OH)D and activated T cells.⁽⁸⁻¹⁰⁾ However, these studies were limited by their study population (small sample size, adult or mouse populations) or lack of adjustment for possible confounders as season of blood draw, herpes seropositivity, and lifestyle determinants. Our results therefore extend observations from previous studies.

Vitamin D levels might have direct effects on memory T cells, because naive T lymphocytes contain 1 α -Hydroxylase (CYP27B) and upregulate VDR expression upon stimulation via the T cell receptor.⁽⁵⁾ The mitochondrial cytochrome P450 enzyme 1 α -Hydroxylase catalyzes the transformation from the inactive form of vitamin D to the active

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form of vitamin D.(20) Binding of this active form of vitamin D to the VDR leads to upregulation of PLC- γ 1, which is a key enzyme in the T cell receptor-signaling pathway.(21) The VDR can bind to promoter regions of various genes, inducing recruitment of transcription factors and co-regulatory molecules on promoters that regulate gene transcription to control cell proliferation, differentiation, apoptosis and angiogenesis.(4) Via these epigenetic mechanisms, vitamin D can enhance antigen responses and maturation of naive T cells.(4) Additionally, vitamin D affects T-cell homing via induction of chemokine receptor CCR10, resulting in increased responsiveness to chemokine CCL27 and the ability for T cells to migrate to sites of infection.(22) We did not observe an association between serum 25(OH)D and CD8TemRO numbers, whereas we did observe these associations for CD4TemRA, CD4TemRO and CD8TemRA. One explanation might be that vitamin D mainly enhances the function of regulatory CD4+T cells.(23) The observed association between 25(OH)D and memory CD4+T cells may therefore partly rely on increased numbers of regulatory CD4+T cells.(23)

Higher serum 25(OH)D levels have been reported to be protective against autoimmune diseases.(1) This could be explained by inhibition of IL-2-gene transcription by the VDR.(24) The cytokine IL-2 is needed by T cells to proliferate.(24) Therefore, immune responses can be regulated by limiting the presence of IL-2.(24) This mechanism could contribute to explain the observed preventive effects of vitamin D on autoimmune diseases. Additionally, vitamin D suppresses cell-mediated Th1 immunity and promotes synthesis of CD4+CD25+T-regulatory lymphocytes that produce the anti-inflammatory cytokine IL-10.(22)

In line, we observed different associations between 25(OH)D and memory T cells for boys and girls, which extends previous literature that describes sex differences in the immune system and immune development.(18) Additionally, it has been reported that males experience infections more frequently than females.(25) Furthermore, sex differences in body

composition could be an explanation because vitamin D is fat-soluble.(26) However, upon correction for URTI and total fat and fat-free mass, no changes in effect estimates were observed. Hence, hormonal differences between boys and girls that already exist in utero and before puberty might explain the observed sex-specific differences of vitamin D on memory T cells.(27)

We did not observe significant associations between 25(OH)D and URTIs at the age of six years. A recent meta-analysis concludes preventive effects of vitamin D supplementation on respiratory infections.(28) Similarly, observational studies have found that higher 25(OH)D levels protect against respiratory infections.(1) These different results could be explained by various reasons. First, our observational design only captures one-year of URTIs preceding vitamin D levels at the age of six years. Second, information on URTIs in our study is obtained from questionnaires, which could introduce bias. Third, the meta-analysis discusses effects of vitamin D supplementation, whereas our study determines the association between serum 25(OH)D and URTIs. Interestingly, this meta-analysis observed the strongest effect in individuals with profound deficient 25(OH)D levels (<25 nmol/L) at baseline.(28) Also, the size of the effect varies according to the causative agent.(1)

In addition, no associations between herpesvirus seropositivity and 25(OH)D were observed in our study. Previous studies on this topic are scarce: an in-vitro study observed down-regulation of the VDR by CMV and it has been hypothesized that EBV is linked to vitamin D status.(29) However, no in-vivo studies in a healthy population of children have been performed on this association.

Methodological considerations

The strength of this study is the large number of children and the embedment in a large population-based prospective study. The prospective design of the Generation R cohort enabled us to adjust for a large number confounders. Another strength is the accurate

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measurement of exposure and outcome. ID-XLC-MS/MS is highly sensitive and specific in measuring 25(OH)D levels and therefore the standard for epidemiological studies.(12) Serum 25(OH)D was measured because this provides the best estimate of vitamin D status in individuals.(30) Furthermore, extensive and detailed immune phenotyping enabled us to evaluate functionally distinct subsets of naive and memory T-lymphocytes. IgG-CMV and IgG-EBV-VCA were associated with significant expansions of the CD8+memory compartment (approximately 2-fold), and to a lesser extent (approximately 1.1 to 1.3-fold) with expansions of CD4+memory T cells in our previous study.(13) These CD4+memory T cells included both CD4TemRO and CD4TemRA cells. Therefore, in our study, we included herpes seropositivity as confounders in the analyses. Additionally, we included relevant sociodemographic, lifestyle confounders. However, residual confounding cannot be excluded.

Conclusions

We observed positive associations between serum 25(OH)D and Tem numbers in children. This indicates that vitamin D may enhance cellular immunity in young children. Since T cell memory is long lasting, these effects of vitamin D in young children might affect the risk of infectious and inflammatory diseases at later age.

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Table 1. Child and Maternal Characteristics of the Study Population stratified by 25(OH)D status

	Serum 25(OH)D			
	Deficient (N=1005) (<50 nmol/L)	Sufficient (N=1129) (50 to <75 nmol/L)	Optimal (N=1055) (≥ 75 nmol/L)	
Child Characteristics				P-value
Sex				0.167
Boy	535 (53.2)	591 (52.3)	520 (49.3)	
Girl	470 (46.8)	538 (47.7)	535 (50.7)	
Age, years	6.07 [5.73, 8.24]	5.98 [5.69, 7.71]	5.96 [5.68, 7.55]	<0.001
Birth weight (g)	3383 (575)	3435 (580)	3424 (561)	0.088
Ethnicity (N,%)				<0.001
Dutch or other Western	446 (44.4)	853 (75.6)	914 (86.6)	
Non-Western	559 (55.6)	276 (24.4)	141 (13.4)	
BMI at 6 years, kg/m ²	16.1 [13.6, 22.6]	15.9 [13.9, 20.6]	15.7 [13.7, 19.9]	<0.001
More than 1 child in household	799 (79.5)	933 (82.6)	900 (85.3)	0.002
Herpes Virus Seropositivity, IgG (N,%)				
CMV	454 (45.2)	393 (34.8)	344 (32.6)	<0.001
EBV	587 (58.4)	566 (50.1)	462 (43.8)	<0.001
HSV-1	274 (27.3)	203 (18.0)	157 (14.9)	<0.001
Season of blood sampling (N,%)				<0.001
Winter	391 (38.9)	242 (21.4)	117 (11.1)	
Spring	341 (33.9)	353 (31.3)	227 (21.5)	
Summer	75 (7.5)	258 (22.9)	409 (38.8)	
Fall	198 (19.7)	276 (24.4)	302 (28.6)	
Playing sports (N,%)	378 (37.6)	540 (47.8)	499 (47.3)	<0.001
Watching television using computer, hours/day	1.82 [0.26; 6.21]	1.32 [0.29, 4.49]	1.29 [0.25, 4.07]	<0.001
Playing outside at daytime, hours/day	0.93 [0.00; 4.22]	1.23 [0.14; 4.30]	1.57 [0.23; 4.49]	<0.001
Upper Respiratory Tract Infections over previous year at 6 years				0.267
Never	723 (71.9)	822 (72.8)	777 (73.6)	
1-2 times	176 (17.5)	194 (17.2)	195 (18.5)	
> 2 times	106 (10.5)	113 (10.0)	83 (7.9)	
25(OH)D level at 6 years, nmol/L	36.0 [12.9; 50.0]	63.0 [51.0; 75.0]	89.0 [76.0; 157.4]	<0.001
Maternal Characteristics				
Folic Acid supplement use during pregnancy (N,%)				0.657
Start Preconceptionally	303 (30.1)	318 (28.2)	325 (30.8)	
Start in first 10 weeks of pregnancy	304 (30.2)	365 (32.3)	323 (30.6)	
None	398 (39.6)	446 (39.5)	407 (38.6)	
Breastfeeding during first 4 months (%)				<0.001
Exclusively Breastfed	174 (17.3)	295 (26.1)	266 (25.2)	
Partially Breastfed	727 (72.3)	723 (64.0)	676 (64.1)	
Never Breastfed	104 (10.3)	111 (9.8)	113 (10.7)	
Smoking during pregnancy (%)				0.009
Never	720 (71.6)	856 (75.8)	812 (77.0)	
Until pregnancy was known	92 (9.2)	111 (9.8)	95 (9.0)	
Continued during pregnancy	193 (19.2)	162 (14.3)	148 (14.0)	
Net monthly household income, euro				<0.001
< 2200	664 (66.1)	446 (41.3)	316 (30.0)	
≥ 2200	341 (33.9)	663 (58.7)	739 (70.0)	

Abbreviations: CMV, cytomegalovirus; EBV, Epstein Barr Virus, HSV-1, Herpes Simplex Virus type 1; 25(OH)D, 25-hydroxyvitamin D. Values are mean $\pm$ SD, median (95% range), or percentages and based on the imputed data. Kruskal Wallis rank sum tests, one-way ANOVA and chi-squared tests were conducted to examine possible differences in baseline characteristics between deficient, sufficient and optimal 25(OH)D levels.

Table 2. Associations of 25(OH)D levels (per 10 nmol/L) with blood lymphocyte cell numbers at 6 years of age

Blood Lymphocytes	β (95% CI)	P-value
Total T	0.718 (-0.176;1.620)	0.12
Total B	-0.068 (-1.166;1.042)	0.90
Total NK	0.300 (1.022;1.622)	0.66
CD4+	0.626 (-0.293;1.554)	0.18
CD4 naive	0.399 (-0.873;1.687)	0.54
CD4 Tcm	-0.752 (-1.940;0.450)	0.22
CD4 TemRA	2.197 (0.535;3.885)	0.009
CD4 TemRO	1.496 (0.381;2.624)	0.008
CD8+	0.891 (-0.175;1.964)	0.10
CD8 naive	0.570 (-0.806;1.964)	0.42
CD8 Tcm	-1.066 (-2.899;0.801)	0.26
CD8 TemRA	1.819 (0.108;3.560)	0.04
CD8 TemRO	0.099 (-1.542;1.757)	0.91

^a N= 3,189, ^b N= 990, ^c N= 986.

Abbreviations: Tcm, Central Memory T lymphocytes; TemRA, Effector Memory RA positive T lymphocytes; TemRO, Effector Memory RO positive T lymphocytes; CI, confidence interval.

Betas (β s) represent regression coefficients derived from multivariable linear regression, based on multiple imputed dataset. The regression coefficients represent the percentage difference in cell number per 10 nmol/L higher 25(OH)D levels. Associations are adjusted for sex, age, birth weight, ethnicity, number of children in household, CMV seropositivity, HSV-1 seropositivity, EBV seropositivity, upper respiratory tract infections (URTIs) over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy. Bold values indicate statistically significant ($P < 0.05$) associations.

Table 3. Associations of 25(OH)D levels (per 10 nmol/L) with blood lymphocyte cell numbers at 6 years of age stratified by sex

	Boys (N=1,646)		Girls (N=1,543)	
Blood lymphocytes	β (95% CI)	P-value	β (95% CI)	P-value
Total T	1.596 (0.323;2.884) ^a	0.014	-0.386 (-1.686;0.929) ^d	0.56
Total B	0.750 (-0.845;2.370) ^a	0.36	-1.195 (-2.731;0.366) ^d	0.13
Total NK	0.728 (-1.158;2.649) ^a	0.45	0.155 (-1.702;2.047) ^d	0.87
CD4+	1.673 (0.372;2.992) ^b	0.012	-0.616 (-1.959;0.744) ^e	0.37
CD4 naive	1.889 (0.058;3.754) ^c	0.043	-1.349 (-3.175;0.512) ^f	0.15
CD4 Tcm	-0.365 (-2.084;1.383) ^c	0.68	-1.312 (-3.015;0.419) ^f	0.14
CD4 TemRA	4.035 (1.760;6.362) ^c	<0.001	0.191 (-2.258;2.703) ^f	0.88
CD4 TemRO	0.932 (-0.653;2.544) ^c	0.25	2.078 (0.448;3.734) ^f	0.012
CD8+	1.364 (-0.155;2.905) ^b	0.08	0.140 (-1.384;1.688) ^e	0.86
CD8 naive	0.838 (-1.112;2.828) ^c	0.40	0.310 (-1.898;1.998) ^f	0.98
CD8 Tcm	-0.554 (-3.193;2.157) ^c	0.69	-1.665 (-4.307;1.005) ^f	0.22
CD8 TemRA	3.150 (0.846;5.506) ^c	0.007	-0.309 (-2.596;2.143) ^f	0.98
CD8 TemRO	0.257 (-1.981;2.546) ^c	0.82	-0.322 (-2.589;2.594) ^f	0.80

^a N= 1,646, ^b N= 509, ^c N= 506, ^d N= 1,543, ^e N= 481, ^f N= 480.

Abbreviations: Tcm, Central Memory T lymphocytes; TemRA, Effector Memory RA positive T lymphocytes; TemRO, Effector Memory RO positive T lymphocytes; CI, confidence interval.

Betas (β s) represent regression coefficients derived from multivariable linear regression, based on multiple imputed dataset. The regression coefficients represent the percentage difference in cell number per 10 nmol/L higher 25(OH)D levels. Associations are adjusted for age, birth weight, ethnicity, number of children in household, CMV seropositivity, HSV-1 seropositivity, EBV seropositivity, upper respiratory tract infections (URTIs) over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy. Bold values indicate statistically significant (<0.05) associations.

Table 4. Associations of 25(OH)D levels (per 10nmol/L) at 6 years of age with childhood infections

	Total N	OR (95% CI)	P-value
Herpesvirus seropositivity			
CMV	1,185	1.001 (0.969;1.034)	0.95
EBV	1,606	0.994 (0.962;1.028)	0.72
HSV-1	630	0.982 (0.944;1.023)	0.39
Upper respiratory tract infection at 6 years			
0	2,017	REF	REF
1-2	343	1.057 (0.999;1.119)	0.06
>2	272	0.956 (0.903;1.012)	0.12

Abbreviations: CMV, cytomegalovirus; EBV, Epstein Barr Virus, HSV-1, Herpes Simplex Virus type 1; OR, odds ratio; CI, confidence interval.

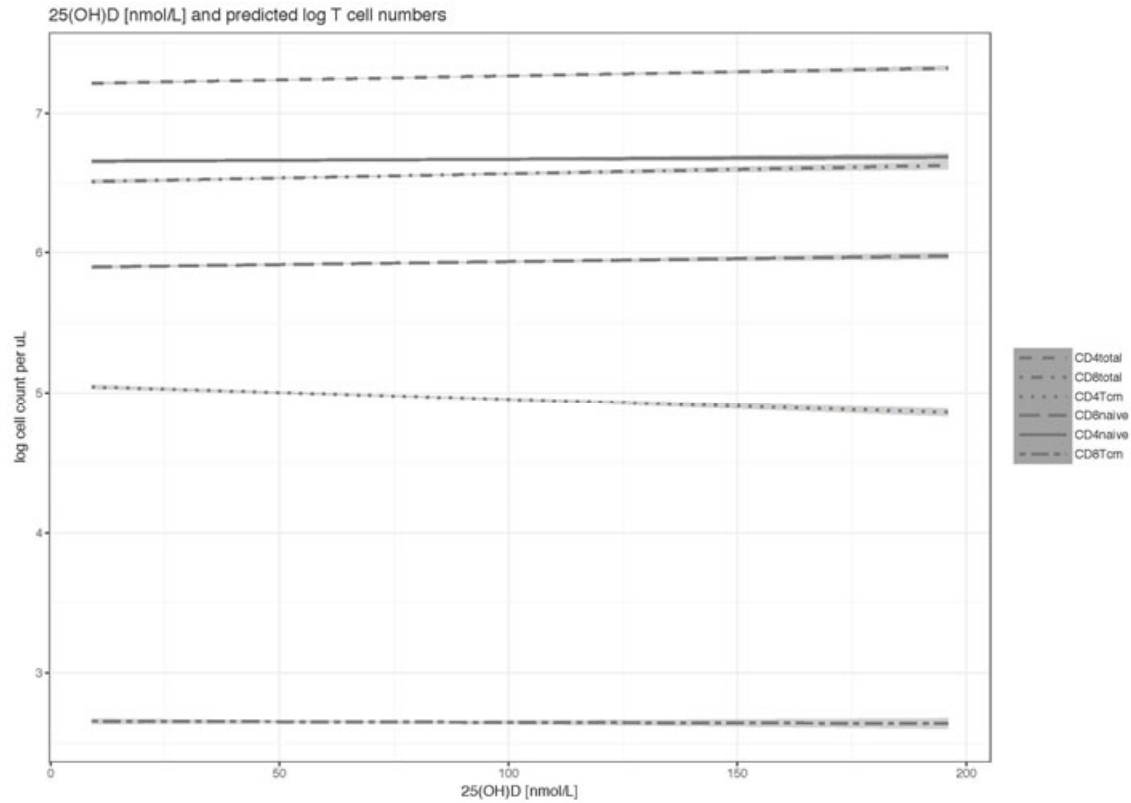
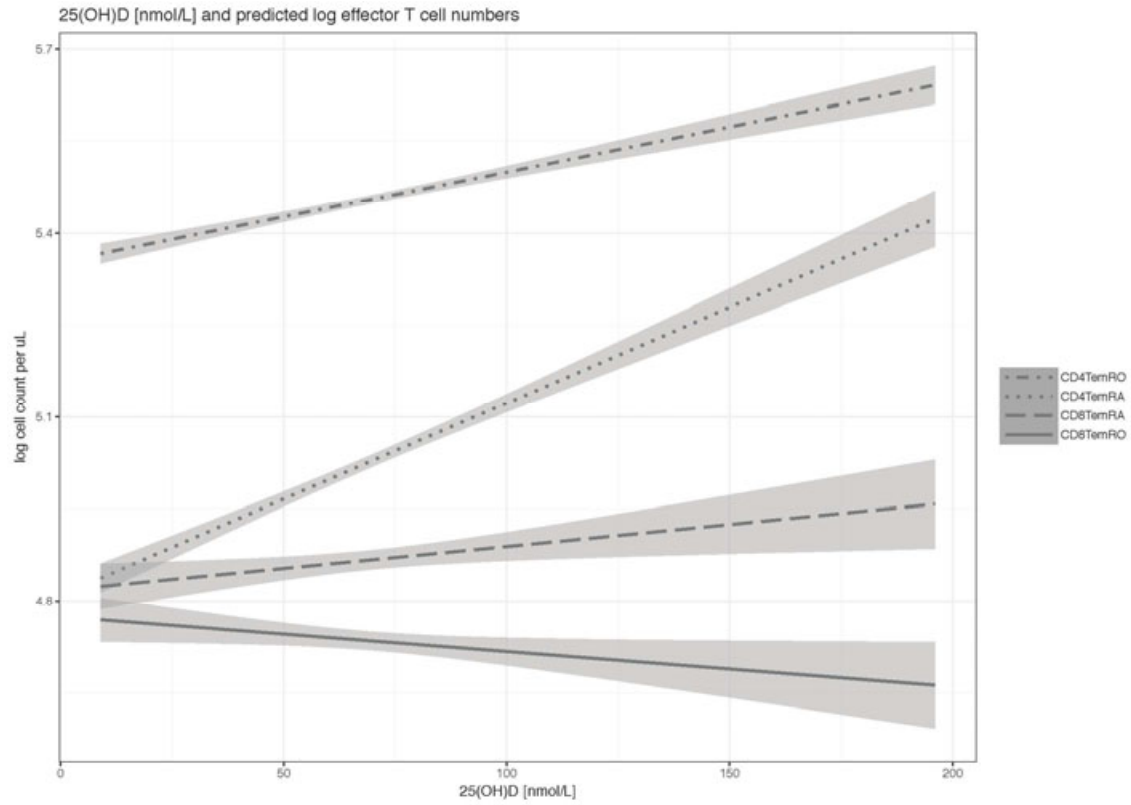
The odds ratios (ORs) are derived from logistic regression analyses, based on multiple imputed dataset. The ORs of herpesvirus seropositivity or upper respiratory tract infections (URTIs) per 10 nmol/L increase in 25(OH)D levels are represented. The following covariates are added to the logistic regression models: sex, birth weight, age, ethnicity, birth weight, number of children in household, CMV seropositivity, HSV-1 seropositivity, EBV seropositivity, URTIs over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy. Bold values indicate statistically significant (<0.05) associations.

Figure 1. The association between 25(OH)D [nmol/L] and predicted log T memory cell numbers

Abbreviations: Tcm, Central Memory T lymphocytes; TemRA, Effector Memory RA positive T lymphocytes; TemRO, Effector Memory RO positive T lymphocytes. Figure 1 presents the association between 25(OH)D and predicted log cell count per μ L. The Figure is adjusted for the following confounders: sex, birth weight, age, ethnicity, birth weight, number of children in household, CMV seropositivity, HSV-1 seropositivity, EBV seropositivity, URTIs over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy.

Figure 2. The association between 25(OH)D [nmol/L] and predicted log T cell numbers

Abbreviations: Tcm, Central Memory T lymphocytes; TemRA, Effector Memory RA positive T lymphocytes; TemRO, Effector Memory RO positive T lymphocytes. Figure 2 presents the association between 25(OH)D and predicted log cell count per μ L. The Figure is adjusted for the following confounders: sex, birth weight, age, ethnicity, birth weight, number of children in household, CMV seropositivity, HSV-1 seropositivity, EBV seropositivity, URTIs over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy. No significant associations were observed between these T cell numbers and 25(OH)D ($p>0.05$).



Supplementary Table 1. Characteristics of the study population based on original data

Child Characteristics (N=3,189)	Total N (%), Median (95% range) or mean (SD)	Missing (%)
Sex (N,%)		0 (0.0)
Boy	1534 (48.4)	
Girl	1646 (51.6)	
Age, years	6.0 (5.7-7.9)	0 (0.0)
Birth weight (g)	3415 (573)	6 (0.2)
Ethnicity (N,%)		118 (3.7)
Dutch or other Western	2155 (67.6)	
Non-Western	916 (32.4)	
BMI at 6 years, kg/m ²	15.9 (13.8-21.2)	5 (0.2)
More than 1 child in household	2240 (83.2)	496 (15.6)
Herpes Virus Seropositivity, IgG (N,%)		
CMV	1185 (37.4)	18 (0.6)
EBV	1606 (50.7)	21 (0.7)
HSV-1	630 (19.9)	18 (0.6)
Season of blood sampling (N,%)		0 (0.0)
Winter	750 (23.5)	
Spring	921 (28.9)	
Summer	742 (23.3)	
Fall	776 (24.3)	
Playing sports (N,%)	1233 (28.7)	457 (14.3)
Watching television using computer, hours/day	1.7 (0.3-5.0)	825 (25.9)
Playing outside at daytime, hours/day	1.1 (0.1-3.6)	819 (25.7)
Upper Respiratory Tract Infections over previous year at 6 years		557 (17.5)
Never	2017 (76.6)	
1-2 times	343 (13.0)	
> 2 times	272 (10.3)	
25(OH)D level at 6 years, nmol/L	63.8 (17.0-131.0)	0 (0.0)
25(OH)D status at 6 years, nmol/L (N,%)		0 (0.0)
Deficient (<50 nmol/L)	1005 (31.5)	
Sufficient (50 to <75 nmol/L)	1129 (35.4)	
Optimal (≥75 nmol/L)	1055 (33.1)	
Maternal Characteristics (N=3,189)	Total N (%) or Median (95% range)	Missing (%)
Folic Acid supplement use during pregnancy (N,%)		1123 (35.2)
Start Preconceptionally	831 (40.2)	
Start in first 10 weeks of pregnancy	618 (29.9)	
None	617 (29.9)	
Breastfeeding during first 4 months (%)		527 (30.5)
Exclusively Breastfed	523 (25.0)	
Partially Breastfed	1371 (65.6)	
Never Breastfed	195 (9.3)	
Smoking during pregnancy (%)		183 (10.6)
Never	1150 (77.4)	
Until pregnancy was known	143 (9.2)	
Continued during pregnancy	254 (13.4)	
Net monthly household income, euro		782 (24.5)
< 2200	982 (40.8)	
≥ 2200	1425 (59.2)	

Abbreviations: CMV, cytomegalovirus; EBV, Epstein Barr Virus, HSV-1, Herpes Simplex Virus type 1; 25(OH)D, 25-hydroxyvitamin D. Values are based on the non-imputed data.

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Times, 11 pt

Supplementary Table 2. Associations of 25(OH)D levels (per 10 nmol/L) with blood lymphocyte cell numbers at 6 years of age stratified by serum 25(OH)D status

Blood Lymphocytes	Deficient 25(OH)D (<50 nmol/L)		Sufficient 25(OH)D (50 to <75 nmol/L)		Optimal 25(OH)D (≥75 nmol/L)	
	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
Total T	-0.029 (-0.090;0.033) ^a	0.36	-0.043 (-0.090;0.005) ^d	0.078	REF	REF
Total B	0.010 (-0.066;0.086) ^a	0.80	-0.023 (-0.082;0.035) ^d	0.44	REF	REF
Total NK	-0.008 (-0.099;0.082) ^a	0.86	-0.007 (-0.077;0.063) ^d	0.84	REF	REF
CD4+	-0.023 (-0.086;0.040) ^b	0.48	-0.057 (-0.106;-0.007)^e	0.024	REF	REF
CD4 naive	0.001 (-0.087;0.088) ^c	0.99	-0.084 (-0.152;-0.016)^f	0.015	REF	REF
CD4 Tcm	0.068 (-0.015;0.150) ^c	0.11	-0.056 (-0.120;0.008) ^f	0.089	REF	REF
CD4 TemRA	-0.163 (-0.276;-0.051)^c	0.005	-0.017 (-0.104;0.07) ^f	0.71	REF	REF
CD4 TemRO	-0.078 (-0.154;-0.002)^c	0.044	-0.014 (-0.073;0.045) ^f	0.64	REF	REF
CD8+	-0.039 (-0.112;0.034) ^b	0.30	-0.015 (-0.071;0.042) ^e	0.60	REF	REF
CD8 naive	-0.017 (-0.112;0.077) ^c	0.72	-0.027 (-0.099;0.046) ^f	0.47	REF	REF
CD8 Tcm	0.114 (-0.014;0.234) ^c	0.08	-0.092 (-0.191;0.006) ^f	0.06	REF	REF
CD8 TemRA	-0.162 (-0.275;-0.045)^c	0.006	0.029 (-0.061;0.119) ^f	0.53	REF	REF
CD8 TemRO	0.20 (-0.093;0.134) ^c	0.73	0.042 (-0.047;0.130) ^f	0.35	REF	REF

^a N= 1005, ^b N= 246, ^c N= 244, ^d N= 1129, ^e N= 351, ^f N= 351.

Abbreviations: Tcm, Central Memory T lymphocytes; TemRA, Effector Memory CD45+RA positive T lymphocytes; TemRO, Effector Memory CD45+RO positive T lymphocytes; CI, confidence interval. Betas (βs) represent regression coefficients derived from multivariable linear regression, based on multiple imputed dataset. The regression coefficients represent the percentage difference in cell number with an optimal 25(OH)D status as reference. Associations are adjusted for gender, age, birth weight, ethnicity, number of children in household, CMV/HSV-1/EBV seropositivity at the age of 6 year, upper respiratory tract infections (URTIs) over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy. Bold values indicate statistically significant (P<0.05) associations.

Formattato: Tipo di carattere: 11 pt

Formattato: Inglese (Stati Uniti)

Formattato: Tipo di carattere: 9 pt

Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

Formattato: Tipo di carattere: 9 pt

Formattato: Tipo di carattere: 9 pt

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Formattato: Inglese (Stati Uniti)

Formattato: Inglese (Stati Uniti)

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Supplementary Table 1. Characteristics of the study population based on the original dataset

Child Characteristics (N=3,189)	Total N (%) or Median (95% range)	Missing (%)
Sex (N,%)		0 (0.0)
Boy	1534 (48.4)	
Girl	1646 (51.6)	
Age, years	6.0 (5.7-7.9)	0 (0.0)
Birth weight (g)	3415 (573)	6 (0.2)
Ethnicity (N,%)		118 (3.7)
Dutch or other Western	2155 (67.6)	
Non-Western	916 (32.4)	
BMI at 6 years, kg/m ²	15.9 (13.8-21.2)	5 (0.2)
More than 1 child in household	2240 (83.2)	496 (15.6)
Herpes Virus Seropositivity, IgG (N,%)		
CMV	1185 (37.4)	18 (0.6)
EBV	1606 (50.7)	21 (0.7)
HSV-1	630 (19.9)	18 (0.6)
Season of blood sampling (N,%)		0 (0.0)
Winter	750 (23.5)	
Spring	921 (28.9)	
Summer	742 (23.3)	
Fall	776 (24.3)	
Playing sports (N,%)	1233 (28.7)	457 (14.3)
Watching television using computer, hours/day	1.7 (0.3-5.0)	825 (25.9)
Playing outside at daytime, hours/day	1.1 (0.1-3.6)	819 (25.7)
Upper Respiratory Tract Infections over previous year at 6 years		557 (17.5)
Never	2017 (76.6)	
1-2 times	343 (13.0)	
> 2 times	272 (10.3)	
25(OH)D level at 6 years, nmol/L	63.8 (17.0-131.0)	0 (0.0)
25(OH)D status at 6 years, nmol/L (N,%)		0 (0.0)
Deficient (<50 nmol/L)	1005 (31.5)	
Sufficient (50 to <75 nmol/L)	1129 (35.4)	
Optimal (≥75 nmol/L)	1055 (33.1)	
Maternal Characteristics (N=3,189)	Total N (%) or Median (95% range)	Missing (%)
Folic Acid supplement use during pregnancy (N,%)		1123 (35.2)
Start Preconceptionally	831 (40.2)	
Start in first 10 weeks of pregnancy	618 (29.9)	
None	617 (29.9)	
Breastfeeding during first 4 months (%)		527 (30.5)
Exclusively Breastfed	523 (25.0)	
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Never Breastfed	195 (9.3)	
Smoking during pregnancy (%)		183 (10.6)
Never	1150 (77.4)	
Until pregnancy was known	143 (9.2)	
Continued during pregnancy	254 (13.4)	
Net monthly household income, euro		782 (24.5)
< 2200	982 (40.8)	
≥ 2200	1425 (59.2)	

Abbreviations: CMV, cytomegalovirus; EBV, Epstein Barr Virus, HSV-1, Herpes Simplex Virus type 1; 25(OH)D, 25-hydroxyvitamin D. Values are based on the non-imputed data.

Supplementary Table 2. Characteristics of the study population stratified by 25(OH)D status.

Blood Lymphocytes	Deficient 25(OH)D (<50 nmol/L)		Sufficient 25(OH)D (50 to <75 nmol/L)		Optimal 25(OH)D (≥ 75 nmol/L)	
	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
Total T	-0.029 (-0.090;0.033) ^a	0.36	-0.043 (-0.090;0.005) ^d	0.078	REF	REF
Total B	0.010 (-0.066;0.086) ^a	0.80	-0.023 (-0.082;0.035) ^d	0.44	REF	REF
Total NK	-0.008(-0.099;0.082) ^a	0.86	-0.007 (-0.077;0.063) ^d	0.84	REF	REF
CD4+	-0.023 (-0.086;0.040) ^b	0.48	-0.057 (-0.106;-0.007)^e	0.024	REF	REF
CD4 naive	0.001 (-0.087;0.088) ^c	0.99	-0.084 (-0.152;-0.016)^f	0.015	REF	REF
CD4 Tcm	0.068 (-0.015;0.150) ^c	0.11	-0.056 (-0.120;0.008) ^f	0.089	REF	REF
CD4 TemRA	-0.163 (-0.276;-0.051)^c	0.005	-0.017 (-0.104;0.07) ^f	0.71	REF	REF
CD4 TemRO	-0.078 (-0.154;-0.002)^c	0.044	-0.014 (-0.073;0.045) ^f	0.64	REF	REF
CD8+	-0.039 (-0.112;0.034) ^b	0.30	-0.015 (-0.071;0.042) ^e	0.60	REF	REF
CD8 naive	-0.017 (-0.112;0.077) ^c	0.72	-0.027 (-0.099;0.046) ^f	0.47	REF	REF
CD8 Tcm	0.114 (-0.014;0.234) ^c	0.08	-0.092 (-0.191;0.006) ^f	0.06	REF	REF
CD8 TemRA	-0.162 (-0.725;-0.045)^c	0.006	0.029 (-0.061;0.119) ^f	0.53	REF	REF
CD8 TemRO	0.20 (-0.093;0.134) ^c	0.73	0.042 (-0.047;0.130) ^f	0.35	REF	REF

^a N= 1005, ^b N= 246, ^c N= 244, ^d N= 1129, ^e N= 351, ^f N= 351.

Abbreviations: Tcm, Central Memory T lymphocytes; TemRA, Effector Memory CD45+RA positive T lymphocytes; TemRO, Effector Memory CD45+RO positive T lymphocytes; CI, confidence interval.

Betas (β s) represent regression coefficients derived from multivariable linear regression, based on multiple imputed dataset. The regression coefficients represent the percentage difference in cell number with an optimal 25(OH)D status as reference. Associations are adjusted for gender, birth weight, age, ethnicity, number of children in household, CMV/HSV-1/EBV seropositivity at the age of 6 year, upper respiratory tract infections (URTIs) over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy. Bold values indicate statistically significant ($P<0.05$) associations.