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ORIGINAL ARTICLE **Epidemiology of Allergic Disease**

Maternal vitamin D intake during pregnancy increases gene expression of ILT3 and ILT4 in cord blood

M. K. Rochat[1]*, M. J. Ege[1]*, D. Plabst[1], J. Steinle[2], S. Bitter[3], C. Braun-Fahrländer[3], J-C. Dalphin[4], J. Riedler[5], M. Roponen[6], M-R. Hirvonen[6,7], G. Büchele[8], H. Renz[9], R. Lauener[2,10], S. Krauss-Etschmann[1,11]*, E. von Mutius[1]* and the PASTURE Study group

[1]Children's Hospital, University of Munich, Munich, Germany, [2]Children's Hospital, University of Zurich, Zurich, Switzerland, [3]Institute of Social and Preventive Medicine at Swiss Tropical Institute. Associated Institute of the University of Basel, Basel, Switzerland, [4]Department of Respiratory Disease, University Hospital of Besançon, Besançon, France, [5]Children's Hospital, Schwarzach, Austria, [6]Department of Environmental Health, National Institute for Health and Welfare, Kuopio, Finland, [7]Department of Environmental Sciences, University of Kuopio, Kuopio, Finland, [8]Institute of Epidemiology, University of Ulm, Ulm, Germany, [9]Department of Clinical Chemistry and Molecular Diagnostics, Philipps University of Marburg, Germany, [10]Hochgebirgsklinik/Children's Allergy and Asthma Hospital, Davos, Switzerland and Christine Kühne Center for Allergy Research and Education, Davos, Switzerland and [11]Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany

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Correspondence:
M. Rochat, Dr von Haunersche Kinderklinik, Lindwurmstrasse 4, 80337 Munich, Germany.
E-mail: mascha.rochat@med.uni-muenchen.de
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Summary

Background Recent studies indicate that prenatal vitamin D intake may protect against the development of atopic diseases in young children. Vitamin D has been shown to induce tolerogenic antigen-presenting cells such as dendritic cells. Whether the allergy-protective potential of prenatal vitamin D is mediated through such mechanisms is, however, unknown.
Objective To evaluate the association between prenatal vitamin D supplementation and tolerogenic antigen-presenting cells in cord blood (CB) as determined by mRNA measurement of immunoglobulin-like transcripts (ILT)3 and ILT4.
Methods A prospective multi-centre birth cohort was established in rural areas of five European countries. Information on maternal exposures including vitamin D intake was collected by questionnaires during pregnancy. The gene expression of ILT3 and ILT4 was analysed by real-time PCR in the CB of 927 children. Maternal vitamin D supplementation was assessed in Finland and France ($n = 349$).
Results Maternal vitamin D supplementation during pregnancy was associated with an increase in the gene expression of ILT3 ($P = 0.012$) and ILT4 ($P < 0.001$). This association remained significant for ILT4 ($P = 0.020$) and showed a positive trend for the gene expression of ILT3 ($P = 0.059$) after multivariate analysis controlling for various confounders.
Conclusions Vitamin D supplementation during pregnancy may increase the mRNA levels of ILT3 and ILT4 in CB. This finding may point towards an early induction of tolerogenic immune responses by maternal vitamin D intake.

Keywords atopic disease, birth cohort, farming environment, ILT3 and ILT4, vitamin D
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The PASTURE study group: Gertraud Weiß, Ellen Üblagger, Claudia Humer, Manuela Rußegger (Austria); Juha Pekkanen, Raija Juntunen, Reetta Tiihonen, Pekka Tiittanen, Kati Huttunen, Suvi Virtanen, Timo Kauppila, Aino Nevalainen, Sami Remes, Tomi-Pekka Tuomainen, Anne Karvonen, Mikko Lappalainen (Finland); Marie-Laure Dalphin, Dominique Vuitton, Renaud Piarroux, Gabriel Reboux, Sandrine Roussel, Bertrand Sudre (France); Michael Kabesch, Susanne Schmid, Sabina Illi, Nicola Korherr, Jon Genuneit, Richard Peter, Serdar Sel, Nicole Blümer, Petra Pfefferle (Germany); Bert Brunekreef, Ulrike Gehring, Gert Dockes (The Netherlands); Felix H. Sennhauser, Susanne Loeliger, Remo Frei, Caroline Roduit (Switzerland).
*Contributed equally to this work.

Introduction

Increasing evidence suggests that gestational exposures interacting with distinct genetic backgrounds influence the development of atopic diseases. Several mechanisms have been proposed, suggesting that a variety of immune cell types and maturational processes may be involved [1, 2].

Recent studies indicate that prenatal, but not postnatal [3], vitamin D intake protects from the development of

wheeze and atopic diseases in 3- [4] and 5- [5, 6] year-old children. These data may point towards different effects of vitamin D on the prenatal and postnatal immune system and possibly the development of atopic diseases. In turn, others have suggested that vitamin D supplementation may be a cause for atopic diseases [7, 8].

Yet, vitamin D, a known immunomodulator, has been shown to induce tolerogenic dendritic cells (DC) [9]. A general feature of tolerogenic DCs is the up-regulation of the two inhibitory receptors immunoglobulin-like transcripts (ILT)3 and ILT4 [10]. The expression of these two receptors results in the inhibition of nuclear factor (NF)-κB activation, a main transcription factor for inflammatory responses [11, 12]. Beyond their ability to induce regulatory T cells (Treg) [13], tolerogenic DCs render naïve T cells anergic, inhibiting their capacity to respond [11]. Tolerogenic DCs are therefore interesting candidates in the development of atopic diseases.

We hypothesized that prenatal vitamin D supplementation could induce tolerogenic DC at birth. To evaluate this hypothesis in an epidemiological setting, we quantified the gene expression levels of ILT3 and ILT4 in cord blood (CB) samples of a population-based birth cohort of farm and reference children.

Materials and methods

Study population

The Protection against Allergy-Study in Rural Environments (PASTURE) is an ongoing multi-centre birth cohort in rural areas of five European countries (Austria, Finland, France, Germany and Switzerland) designed to assess the role of exposure to various microbial products for the development of childhood asthma and allergies. The aims of the study as well as the study design have been described in detail elsewhere [14]. Briefly, women were contacted in the third trimester of pregnancy. Women who lived or worked on family-run farms where any kind of livestock was kept were assigned to the farm group. The reference group was composed of women from the same rural areas not living or working on a farm. Exclusion criteria were living on farms without livestock, maternal age below 18 years, twin pregnancies, home births, premature deliveries, genetic disease in the offspring, absent telephone connection, insufficient knowledge of the country's language, intention to move away from the study area and commuting to a metropolitan area. The main exposure categories were identified through comprehensive questionnaires and interviews derived from internationally validated questionnaires. Maternal and paternal variables were assessed during the third trimester of pregnancy. Supplementation with vitamin D during pregnancy was assessed by the following question: 'Do you currently take any vitamin, minerals or other dietary supplements? Vitamin D yes/no,' followed by a list of other vitamins and multivitamin preparations. Vitamin D supplementation was only analysed in the Finnish and the French populations as they were the only two populations included in the PASTURE study with recommendations for vitamin D supplementation during pregnancy (Finland: 10 μg of vitamin D supplementation per day between November and the end of March. France: a single parenteral dose of 2500 μg of vitamin D is recommended in the seventh month of pregnancy). Birth and early childhood variables were assessed when the child was 2 months of age. The study was approved by the national ethical boards of the five study centres and informed consent was obtained from the children's parents for questionnaires and blood samples.

Blood sampling

Blood samples were collected from the umbilical cord at birth. For the assessment of mRNA, the blood was collected in a PAXgene® Blood RNA tube containing an RNA-stabilizing solution (PreAnalytiX/Qiagen, Hilden, Germany) and then frozen to −80 °C within 24 h. In a central laboratory (Zurich, Switzerland), the RNA was isolated using the PAXgene 96 Blood RNA Kit (PreAnalytiX/Qiagen) supplemented with RNase-free DNase (Qiagen). The RNA was then quantitatively measured using the NanoDrop ND-1000 UV/VIS-Spectralphotometer (PeqLab Biotechnologie GmbH, Erlangen, Germany). Next, the mRNA was retro-transcribed into cDNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Weiterstadt, Germany). A pre-developed assay with quantitative real-time PCR (TaqMan, Applied Biosystems, Rotkreuz, Switzerland) was used to measure gene expression levels for ILT3 and ILT4 (Applied Biosystems; Hs00275975_m1, Hs00429000_m1), as well as for two housekeeping genes (18S-ribosomal RNA; Applied Biosystems; Hs99999901_s1 and β-2-microglobulin; Applied Biosystems; B2M-Hs99999907_m1) as endogenous controls. From the values of the respective target genes, the geometric mean threshold cycle (C_t) value of the two housekeeping genes was subtracted, resulting in delta C_t values. Calibrating the delta C_t values on the first reference child with detectable C_t values for all genes produced delta C_t values, which were used for statistical analysis [15, 16].

Allergen-specific IgE against 20 common inhalant and food allergens was measured using the Allergy Screen test panel for atopy (Mediwiss Analytic, Moers, Germany) in a central laboratory (Marburg, Germany). This test panel has previously been validated against the *in vitro* IgE CAP system (Pharmacia, Freiburg, Germany) [17].

After stimulation of whole CB cells at the local study centres with a standardized protocol that has been described elsewhere [18], cytokines were measured in the

supernatants in one central laboratory (Marburg, Germany). Briefly, after stimulation with phorbol 12-myristate 13-acetate (5 ng/mL) and ionomycin (1 mg/mL) (P/I) (both from Sigma, Deisenhofen, Germany) for 24 and 48 h at 37 °C, cell-free supernatants were obtained by centrifugation. Cytokine concentrations were measured using ELISA techniques (Opteia, BD, Heidelberg, Germany) according to the manufacturer's instructions. The French study group did not contribute to the cytokine data at birth.

Statistical analyses

Statistical analysis was performed using SAS 9.2 (The SAS Institute, Cary, NC, USA).

The frequencies of the population characteristics were compared between farm and reference children using the χ^2-test for nominal or categorical variables and the *t*-test for continuous variables. β estimates from linear regression models are given with 95% confidence intervals (CI). Reported *P*-values are two-sided. *P*-values < 0.05 were considered significant. Homogeneity of effects was tested by adding multiplicative interaction terms to the regression models following a pre-specified protocol.

In epidemiological studies, adjustment of *P*-values for multiple testing is not performed usually 'because it will lead to fewer errors of interpretation when the data under evaluation are not random numbers but actual observations on nature.' [19]

mRNA data were analysed as continuous values after log transformation of the delta C_t values. This resulted in a satisfactory approximation to a normal distribution.

Vitamin D, farming status, gender as well as the other variables analysed were selected due to their known association with atopic diseases as well as their potential immunomodulatory function. As $< 3\%$ of the total mRNA values were below the detection limit, the analyses were performed with multiple linear regressions models including all observations according to a pre-specified analysis protocol. Statistical methods for censored data such as Tobit regression models were explored in sensitivity analyses, but did not reveal major differences.

Specific CB IgE levels were dichotomized at their detection limit of 0.2 IU/mL. Levels of cytokine production after P/I stimulation were standardized on the number of leucocytes. Log transformation of the detectable values gave satisfactory approximation to a normal distribution. The analysis was performed using a multivariable Tobit regression model where left censored cytokine values were taken into account.

Results

Of the 2871 pregnant women contacted for the PASTURE study, 62% were eligible, and of these 1133 agreed to participate in the study [14]. CB samples were available for the gene expression analysis in 927 subjects (Fig. 1). Vitamin D supplementation during pregnancy differed significantly between the countries [Finland: 53 (28.2%), France: 26 (16.2%), Germany: 11 (5.9%), Switzerland: 2 (1.0%), and Austria: 1 (0.6%), *P*-value = < 0.001], but did not differ between farm and reference mothers (*P*-value = 0.346). In Germany, Switzerland and Austria, further analyses on vitamin D supplementation were not possible because of low exposure frequencies. Therefore, all vitamin D supplementation analyses were performed in the Finnish and the French populations. Table 1 shows the general characteristics of mothers taking vitamin D supplementation in these two populations. In the Finnish and the French populations, children with and without CB mRNA measurements did not differ, with the exception of a lower proportion of caesarean section in the children with mRNA measurements (10% vs. 29%, $P = 0.025$).

When analysing the associations between vitamin D supplementation during pregnancy and the mRNA levels of ILT3 and ILT4 in Finland and France, higher levels were seen for ILT3 ($\beta = 0.18$, $P = 0.059$) and ILT4 ($\beta = 0.21$, $P = 0.020$) in neonates whose mothers had supplemented vitamin D during pregnancy (Fig. 2). The association was independent of farming status, gender and centre as well as all potential confounders with a *P*-value below 0.15 in Table 1 (gestational age, birth weight, primigravida, maternal age, season of birth and current smoking of mothers). Interestingly, vitamin D was also significantly associated with gestational age and birth weight in a bivariate analysis (Table 1). After adjustment for potential confounders, however, the association between vitamin D and gestational age was no longer significant ($\beta = 0.06$, $P = 0.721$), whereas the relation with birth weight remained highly significant ($\beta = 161.64$, $P = 0.005$).

In the Finnish population, vitamin D supplementation occurred more frequently during the winter months as

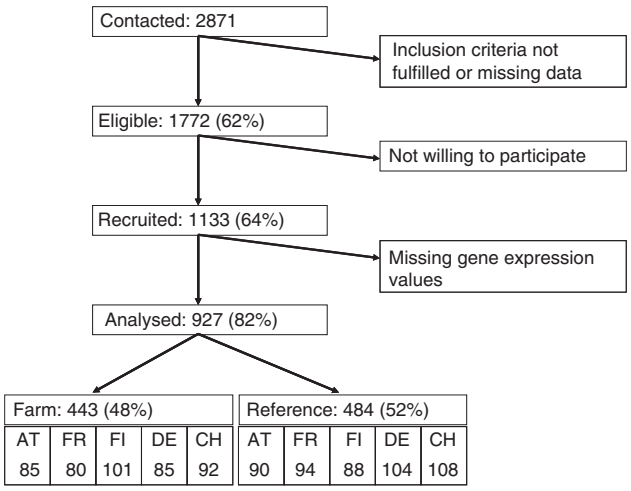

Fig. 1. Recruitment of the study population. AT, Austria, FR, France, FI, Finland, DE, Germany, CH, Switzerland.

Table 1. Population characteristics

	Maternal intake of vitamin D during pregnancy in the Finnish and French population		
	Yes ($n = 79$) No. percentage	No ($n = 270$) No. percentage	*P*-value
Female sex	43 [54.00%]	134 [49.63%]	0.453
Gestational age (weeks)*	40 [1.2]	40 [1.09]	**0.117**
Birth weight (g)*	3720 [523]	3543 [449]	**0.002**
Mode of birth			
Spontaneous	64 [82.05%]	227 [84.70%]	0.290
Vaginal with complications	7 [8.97%]	12 [4.48%]	
Caesarean section	7 [8.97%]	29 [10.82%]	
Pregnancy complications	7 [9.00%]	35 [12.96%]	0.324
Season of birth			
Dec/Jan/Feb	30 [37.97%]	65 [24.07%]	**< 0.001**
Mar/Apr/May	30 [37.97%]	72 [26.67%]	
Jun/Jul/Aug	12 [15.19%]	47 [17.41%]	
Sept/Oct/Nov	7 [8.86%]	86 [31.86%]	
Primigravida	23 [29.00%]	47 [17.41%]	**0.022**
Child with more than two siblings	25 [32.00%]	107 [39.63%]	0.198
Maternal age			
< 30 years old	37 [46.84%]	102 [37.78%]	**0.009**
Between 30 and 35 years old	34 [43.04%]	96 [35.56%]	
Older than 35	8 [10.13%]	72 [26.67%]	
Mother smoking currently	3 [4.00%]	31 [11.48%]	**0.043**
Parental atopy	58 [81.00%]	207 [84.84%]	0.386
Mother living or working on a farm	34 [43.00%]	138 [51.11%]	0.207
Exposure to pets during pregnancy	55 [70.00%]	171 [63.33%]	0.304
Exposure to a stable during pregnancy	37 [47.00%]	130 [49.62%]	0.735
Farm milk consumption during pregnancy	27 [34.00%]	101 [37.41%]	0.600
Mother lived on a farm during childhood	38 [48.00%]	113 [41.85%]	0.324

*Mean (standard deviation). Significant *P*-values (< 0.05) are in bold.

The frequency is compared between mothers with and without vitamin D intake using Pearson's χ^2-test for categorical variables and the *t*-test for continuous variables.

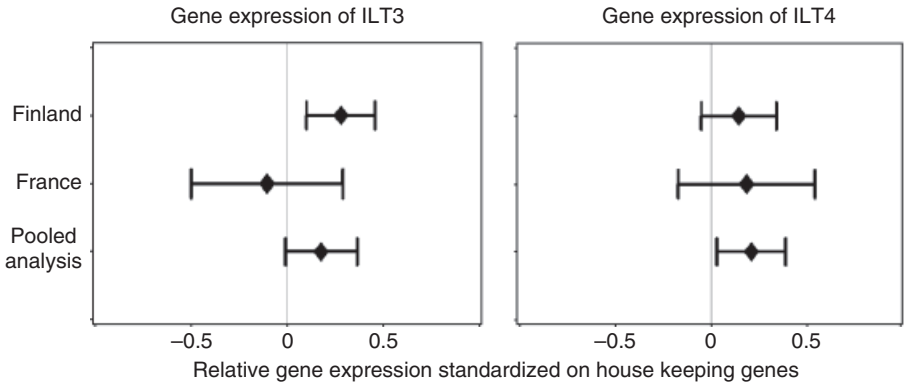

Fig. 2. Association between vitamin D intake during pregnancy and gene expression of ILT3 and ILT4 in Finland and France ($n = 349$). β-estimates and 95% confidence intervals (CI) are shown. A β-estimate greater than 0 indicates a higher gene expression of ILT3 and ILT4, whereas a β-estimate smaller than 0 indicates a lower gene expression of ILT3 and ILT4 in children whose mother supplemented vitamin D during pregnancy. An association with a CI including 0 is not statistically significance at a 0.05 significant level. The models were adjusted for gender, farming status, gestational age, birth weight, primigravida, maternal age, season of birth, current smoking of mothers, and in the pooled analysis, additionally for centre. Reported *P*-values are two-sided.

compared with the summer months (winter months 37.4%, summer months 17.9%; $P = 0.003$). Because of a lower frequency of vitamin D supplementation in the French population, the seasonal distribution could not be assessed. As vitamin D effects strongly depend on sunlight exposure, we additionally analysed the direct association

between season of birth (Winter = December–February; Spring = March–May; Summer: June–August; Autumn: September–November) and ILT3 or ILT4. For ILT3, no association was found ($P = 0.2023$), and for ILT4 the association was significant ($P = 0.001$). However, the association between farming or vitamin D supplementation on ILT4 was not substantially modified by season of birth (change in estimate < 10%).

The mRNA levels of ILT3 and ILT4 differed between farm and reference children, with considerable heterogeneity across the countries as determined by a *P*-value for homogeneity of effects of 0.01 for ILT3 and 0.009 for ILT4. In Austria, Germany and France, gene expression was increased among farm children, whereas in Switzerland, farm children had lower levels than reference children. In Finland, mRNA levels did not vary between farm and reference children. Heterogeneity of associations of ILT3 and ILT4 with farming was formally shown between Switzerland and the other countries (ILT3: *P*-value 0.002, ILT4: *P*-value 0.004), but not between the remaining countries (data not shown). In Switzerland, an inverse association was seen between mRNA levels of ILT3 ($\beta = -0.28$, $P = 0.066$) and ILT4 ($\beta = -0.14$, $P = 0.069$) in the CB of farm children as compared with reference children. In turn, in all countries other than Switzerland, significant increases in the mRNA levels of ILT3 (ILT3: $\beta = 0.14$, $P = 0.014$) and ILT4 ($\beta = 0.15$, $P = 0.003$) were seen in the CB of farm children as compared with reference children. These analyses were adjusted for gender, vitamin D supplementation during pregnancy and, in the pooled analysis with all countries except Switzerland, for centre. In all countries other than Switzerland, farm and reference children differed substantially in many aspects. However, none of the prenatal exposures assessed explained the farming effect on the mRNA levels of ILT3 and ILT4.

In boys, mRNA levels were lower for ILT4 ($\beta = 0.09$, $P = 0.032$) and for ILT3 (β: 0.10, $P = 0.059$) than in girls across all countries and independently of farming, centre or supplementation with vitamin D during pregnancy.

No association was found between specific IgE to inhalant or food allergens in CB and the mRNA levels of ILT3 or ILT4 either in all the countries together (Fig. 3) or in Finland and France (data not shown). Furthermore, no association was found between the mRNA levels of ILT3 or ILT4 and the production of the cytokines IL-12, IL-5, TNFα and IFNγ (data not shown). IL-10 production, however, was inversely associated with mRNA levels of ILT3 and ILT4 after 24 and 48 h of stimulation (Fig. 4). When IL-10 production was dichotomized at the detection limit of 11.4 pg/mL, similar results were obtained for mRNA levels of ILT3 and ILT4 (data not shown).

Discussion

The present analysis revealed a positive association between maternal vitamin D supplementation during pregnancy and mRNA levels of ILT3 and ILT4 in CB. Additionally, prenatal exposure to a farming environment in some countries and female gender in all countries were found to be positively associated with mRNA levels of ILT3 and ILT4. The ILT3 and ILT4 mRNA levels were inversely associated with IL-10 production after stimulation. No relation was found either with IL-5, IL-12, TNF-α, IFN-γ or with CB IgE.

ILT3 and ILT4 transcripts were quantified in whole CB. Therefore, the cellular origin of the transcripts is unknown. However, it has been shown that ILT3 and ILT4 are expressed on DCs, macrophages, monocytes and endothelial cells [10, 20]. As macrophages and endothelial cells are not present in peripheral blood, the mRNA of the

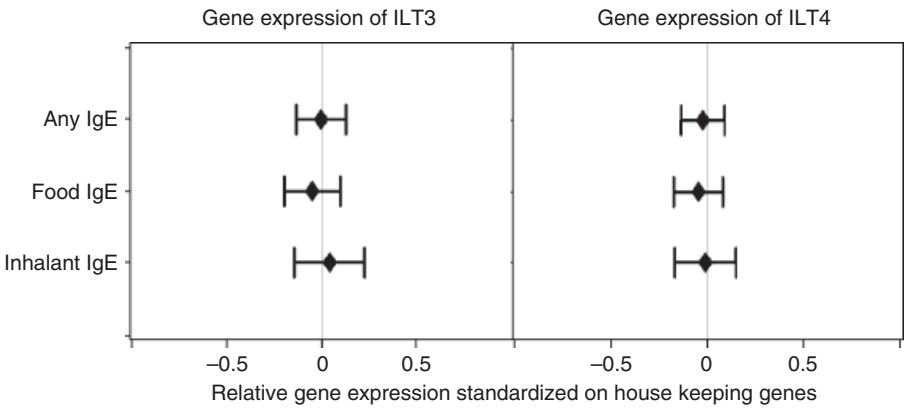

Fig. 3. Association between IgE levels and gene expression of ILT3 and ILT4 ($n = 843$). β-estimates and 95% confidence intervals (CI) are shown. A β-estimate greater than 0 indicates a higher gene expression of ILT3 and ILT4, whereas a β-estimate smaller than 0 indicates a lower gene expression of ILT3 and ILT4. An association with a CI including 0 is not statistically significant at a 0.05 significant level. The models were adjusted for farming status, gender, vitamin D intake during pregnancy and centre. Reported *P*-values are two-sided.

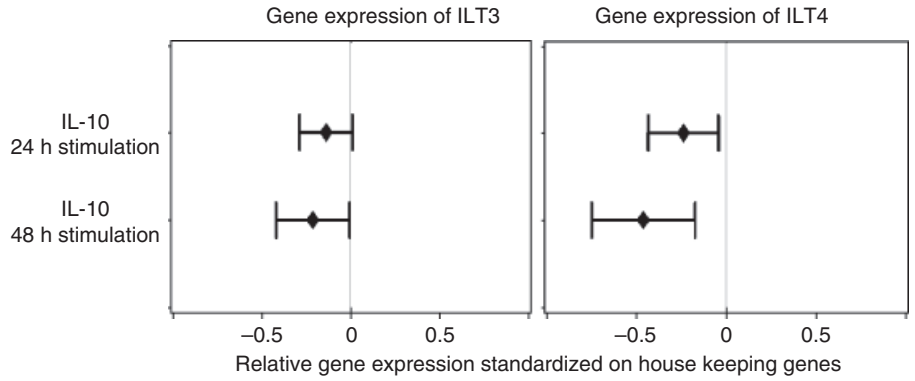

Fig. 4. Association between IL-10 cytokine levels and gene expression of ILT3 and ILT4 ($n = 522$). Continuous IL-10 cytokine values are shown after 24 and 48 h, respectively, after stimulation with P/I. β-estimates and 95% confidence intervals (CI) are shown. A β-estimate greater than 0 indicates a higher gene expression of ILT3 and ILT4, whereas a β-estimate smaller than 0 indicates a lower gene expression of ILT3 and ILT4. An association with a CI including 0 is not statistically significant at a 0.05 significant level. The models were adjusted for farming status, gender, vitamin D intake during pregnancy and centre. Reported *P*-values are two-sided.

two inhibitory receptors is most likely derived from monocytes and DCs.

The association of vitamin D supplementation with markers of tolerogenic DCs is interesting because the activated form of vitamin D, $1,25(OH)_2D3$, has pronounced immunoregulatory properties [7–9]. The biologic effects of $1,25(OH)_2D3$ are mediated by the vitamin D receptor, which is expressed on antigen-presenting cells such as DCs [21]. A number of studies have shown that *in vitro* treatment with vitamin D induces tolerogenic DC [22], up-regulates ILT3 [10] and – in combination with dexamethasone – up-regulates ILT4 [23]. ILT3 has been suggested to act as a master switch in the regulation of antigen-specific responses mediated by CD8+and CD4+T-cells in transplantation as well as cancer immunology, autoimmunity and allergy [24]. The tolerogenic effects of $1,25(OH)_2D3$ and its analogues have been observed on murine DCs *in vitro* and *in vivo* [25].

Despite an established link between maternal vitamin D intake during pregnancy and a reduced risk of wheeze in early childhood [4–6] as well as emerging data on an association between vitamin D intake and food allergy [26], only a few studies have investigated the effects of prenatal vitamin D on the human fetal immune system. One study reported a suppression of Th1 as well as Th2-associated cytokine production after *in vitro* treatment of human CB with $1\alpha,25(OH)2D3$ [27], thereby supporting the idea of an immunomodulatory effect of vitamin D on the human developing immune system. Another study found a positive association between human CB IL-10 and vitamin D levels [28]. This finding is contrary to what may be expected from our data. Both studies, however, are not comparable at many levels. One major difference is that we did not measure IL-10 levels directly after exposure to vitamin D as in the study by Zittermann et al. [28] but measured the cellular capacity to produce IL-10 after stimulation with P/I in neonates whose mother had or had not been exposed to vitamin D. The inverse association between ILT3 and ILT4 mRNA levels and IL-10 found in our study should, nevertheless, be interpreted with caution because it is not in line with other studies. For example, the study by Pedersen et al. [23] showed a positive association between ILT3 and ILT4 gene expression and IL-10 production by vitamin D-stimulated DCs.

As a potential predictor for the development of atopic diseases, we assessed whether the detection of specific IgE antibodies was associated with ILT3 and ILT4 mRNA levels. No association was found. Although high total IgE levels in CB have been shown to be risk factors for the development of allergic diseases [29], $< 5\%$ of the neonates in the PASTURE study had IgE levels above 0.35 kU/L [30]. As the predictive value of specific IgE in CB for the incidence of allergic illnesses later in life is currently unknown, subsequent analyses of the PASTURE cohort are needed to determine the significance of this lack of association.

Findings from a recently published Finnish birth cohort showed an inverse association between maternal vitamin D intake during pregnancy and the risk of asthma and allergic rhinitis in 5-year-old children [6]. In this more urban population, enriched for susceptibility to type 1 diabetes, the effects were linked to common dietary intake of vitamin D and not to supplementary intake. Therefore, it remains to be seen whether ILT3 or ILT4 mRNA levels will be predictive of atopic disease.

A number of epidemiological studies have shown that prenatal and early childhood exposure to a farming environment protect from the development of atopic diseases [31, 32]. Different pathways by which farm-related environmental exposures might affect immune responses have been suggested [1, 33–35] but the mechanisms remain unclear. We assessed whether ILT3 and ILT4 mRNA levels were associated with prenatal

exposure to a farming environment and found a positive association in most study areas.

Although previous studies have shown that the protective farming effect is mediated by specific farm characteristics [36], in the present analysis, none of the assessed individual farm-related exposures accounted for the different gene expression levels between farm and reference children in all countries except for Switzerland. Unknown exposures not covered by the questionnaires may therefore be relevant for ILT3 and ILT4 gene expression. Sunlight exposure, which was not assessed by the questionnaire, might be such an exposure. Farmers are regularly exposed to sunlight during various outdoor activities and sunlight is known to induce the production of vitamin D in the skin [28, 37]. However, if sunlight would account for the farming effect, the levels of ILT3 and ILT4 expression should show seasonal fluctuations and confound the association between farming and ILT3 or ILT4 gene expression. Even though the child's season of birth was significantly associated with ILT4 expression, it did not change the estimate of the associations between farming or vitamin D with ILT4 expression. We therefore suggest that the observed farm effect cannot merely be attributed to increased sun exposure of pregnant farming women.

As in many multi-centre studies, heterogeneity was observed between study centres. Contrary to the other countries, Swiss farm children had lower levels of ILT3 and ILT4 compared with their reference children. Although the protective effect of living on a farm has clearly been shown in Switzerland for specific IgE and clinical symptoms of allergic rhinitis [38], no effect was seen for asthma [36]. Furthermore, maternal exposure during pregnancy was only weakly associated with protection from atopic sensitization [35]. Therefore, farm exposures in Switzerland may differ from other countries. It remains to be seen whether the centre heterogeneity in the CB gene expression of ILT3 and ILT4 will lead to differences in disease manifestation in the different countries.

Boys had lower levels of ILT3 and ILT4 mRNA than girls at birth. This underlines the potential biological relevance of ILT3 and ILT4 expression. However, whether the higher incidence of atopic sensitization and atopic disease observed in boys during the first years of life [39, 40] is related to this finding remains to be elucidated.

The main strengths of the PASTURE study are a well-defined prospective birth cohort study population and well-specified laboratory outcomes. However, some potential limitations exist. The gene expression association with vitamin D could only be assessed in Finland and with some limitations in France. The association with vitamin D remained after controlling for different factors including gestational age, birth weight, primigravida, maternal age, season of birth and current smoking of mothers. Therefore, major confounding by these factors is unlikely. In Finland, vitamin D supplementation during pregnancy is officially recommended. The current recommendations for pregnant women are 10 μg (400 IU) of vitamin D supplementation per day between November and the end of March. Every pregnant woman should therefore supplement vitamin D during 2–5 of her pregnancy months. The questionnaires, which were administered at recruitment at the end of pregnancy, assessed current oral vitamin D supplementation. This partly explains the low (28%) overall point prevalence of vitamin D supplementation in Finland. Nevertheless, the recommendation is reflected in our data, with more women supplementing vitamin D during the winter months than during the summer months. Moreover, the percentage of mothers taking vitamin D supplementation is similar to that of another Finnish birth cohort [6], which strengthens the plausibility of the questionnaire-based answers. Quantification of 25(OH)Vitamin D3 would have reduced non-differential misclassification, which would have strengthened the association and minimized potential bias towards the null. However, in our analysis, the association was still detectable with a relevant and significant estimate. Furthermore, the mothers answered the questionnaire blindly because at the time of data collection (before birth) they were unaware of the ILT3 or ILT4 levels.

In France, a single intramuscular dose of 2500 μg (100 000 IU once) of vitamin D is recommended in the seventh month of pregnancy. Thus, the question on oral supplementation may have led to underreporting of vitamin D supplementation in the French population. In the other countries, there are no recommendations for vitamin D supplementation during pregnancy. Hence, specific vitamin D supplementation was only reported sporadically in the other centres rendering the assessment of a potential association of vitamin D with gene expression of ILT3 and ITL4 in Germany, Austria and Switzerland impossible. In Austria and Switzerland, multivitamin supplementation was reported more frequently. However, multivitamin supplementation was not associated with either ILT3 or ILT4 (data not shown). Additionally, as we did not assess the specific formulations of multivitamins in the different countries, we do not consider multivitamin supplementation an adequate substitute for vitamin D intake. Apart from cod-liver oil preparations, no other dietary sources of vitamin D were assessed in the questionnaire. Cod-liver oil intake was reported scarcely, rendering association analyses for gene expression impossible.

In summary, the present analysis of the PASTURE birth cohort showed vitamin D supplementation during pregnancy to be associated with an increased level of CB gene expression of ILT3 and ILT4, two hallmarks of tolerogenic DCs. Exposure to a farming environment and female gender were independent determinants of increased CB gene expression of ILT3 and ILT4. These factors may

therefore be involved in the induction of tolerogenic DCs and consequently of Tregs, ultimately impacting on the Th1/Th2 balance. Whether this hypothesis can be extended to disease manifestation, and possibly to its prevention, will be left to the follow-up of the PASTURE birth cohort, when, at school age, atopic disease will have become manifest.

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