DIETARY SUPPLEMENTATION WITH HIGH DOSES OF REGULAR VITAMIN D₃ SAFELY REDUCES DIABETES INCIDENCE IN NOD MICE WHEN GIVEN EARLY AND LONG-TERM

Tatiana Takiishi,¹ Lei Ding,¹ Femke Baeke,¹ Isabella Spagnuolo,² Guido Sebastiani,² Jos Laureys,¹ Annemieke Verstuyf,¹ Geert Carmeliet,¹ Francesco Dotta,² Tom L. Van Belle,¹ Conny Gysemans,¹ Chantal Mathieu¹

¹Clinical and Experimental Endocrinology (CEE), Department of Clinical and Experimental Medicine, KU LEUVEN, Campus Gasthuisberg O&N I Herestraat 49 – box 902, 3000 Leuven, Belgium

²Diabetes Unit, Department of Medicine, Surgery and Neurosciences, University of Siena and Fondazione Umberto Di Mario ONLUS – Toscana Life Science Park, Siena, Italy

C.G. and C.M. share senior authorship

Running title:
Lifelong vitamin D₃ reduces diabetes in NOD mice

Word count: abstract (200), main document (3991)

Number of tables: 1

Number of figures: 7
Corresponding author and to whom reprint requests should be addressed:

Chantal Mathieu, M.D., Ph.D.
Clinical and Experimental Endocrinology (CEE)
KU Leuven, Campus Gasthuisberg
O&N I Herestraat 49 – box 902
3000 Leuven, Belgium
E-mail: chantal.mathieu@uzleuven.be
Tel: +32 16 3 46023
Fax: +32 16 3 30718
ABSTRACT

High doses of the active form of vitamin D$_3$, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) prevent diabetes in the non-obese diabetic (NOD) mouse but also elicit unwanted calcemic side-effects. Because immune cells themselves can convert vitamin D$_3$ into 1,25(OH)$_2$D$_3$ locally, we hypothesized that dietary vitamin D$_3$ can also prevent disease. Thus, we evaluated whether dietary administration of high doses of regular vitamin D$_3$ (800 IU per day) during different periods of life (pregnancy and lactation, early-life (3-14 weeks of age), or lifelong (3-35 weeks of age)) safely prevents diabetes in NOD mice. We found that only lifelong treatment raised serum 25-hydroxyvitamin D$_3$ from 173 nmol/L in controls to 290 nmol/L, without inducing signs of calcemic or bone toxicity, and significantly reduced diabetes development in both male and female NOD mice. This diabetes protection by vitamin D$_3$ correlated with preserved pancreatic insulin content and improved insulitis scores. Moreover, vitamin D$_3$ treatment decreased interferon-γ-positive CD8$^+$ T-cells and increased CD4$^+$CD25$^+$FoxP3$^+$ T-cells in pancreatic draining lymph nodes. In conclusion, this study shows for the first time that high doses of regular dietary vitamin D$_3$ can safely prevent diabetes in NOD mice when administered lifelong, although caution is warranted with regards to administering equivalently high doses in humans.
Type 1 diabetes is recognized as an autoimmune-mediated disorder with a variable prodromal phase characterized by the progressive loss of the insulin-producing beta-cells in the pancreatic islets in genetically at-risk individuals (1,2). Several facts support a critical role for environmental factors that trigger the development of type 1 diabetes. Leading environmental candidates include exposure to enteroviruses, early introduction of wheat, and insufficient vitamin D levels. The prevalence of type 1 diabetes increases with latitude of residence and decreased sunlight exposure, whereas exposure to UV light, known to induce vitamin D production in the skin, is associated with the lower incidence of type 1 diabetes in countries closer to the equator (3,4). These observations strengthen the hypothesis that an inadequate vitamin D status due to insufficient sun exposure, dietary uptake and/or abnormalities in its metabolism, may increase the risk of type 1 diabetes (5). Vitamin D deficiency indeed increases the onset and severity of autoimmune type 1 diabetes in at risk children (6) and also in the non-obese diabetic (NOD) mouse (7,8). Moreover, most epidemiological data, based on dietary questionnaires, suggested that vitamin D supplementation during pregnancy, infancy or early adulthood may be associated with a reduced risk of type 1 diabetes later in life (6,9,10). It has been suggested that vitamin D in early life is important for gut maturation, thereby reducing permeability for agents/proteins that can act as potent antigenic stimuli (11,12).

In spite of these exciting results, several areas of controversy remain in this domain and randomized, double-blinded, placebo-controlled trials with extended observation periods in humans are limited. Moreover, most of the human studies have several shortcomings, including recall bias, absence of repeated 25(OH)D measurements, and lack of a quantifiable evaluation of intake of vitamin D from food or supplements, or data on sunlight exposure. So far, convincing evidence is missing on whether dietary vitamin D as either D₂ (ergocalciferol, from plants) or D₃ (cholecalciferol, from animals) can reduce or prevent type 1 diabetes. Moreover, it remains unclear what formulation, dose, route or duration of treatment is ideal to intervene in the diabetes process. In a small pilot study from our group, NOD mice treated with a daily dose of 1,000 international units (IU) (25 µg) of
cholecalciferol (given i.p. from 3 until 70 days of age) were unaffected in their diabetes presentation (13), while the active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃, 5 µg/kg given i.p. on alternative days) from weaning until 200 days of age clearly reduced the development of autoimmune diabetes, although this inhibition occurred only at doses that also caused mild hypercalcemia and bone demineralization (7,14). In another study, oral administration of 50 ng of 1,25(OH)₂D₃ per day reduced diabetes incidence compared to vitamin D-deficient NOD mice (8).

In the present study, we evaluated the effects of high-dose oral vitamin D₃ supplementation administered at various intervals in life – during pregnancy and neonatal life as well as during infancy (early-life) and adulthood (lifelong) – in terms of safety, immune modulation and the development of diabetes in already vitamin D-sufficient NOD mice.
RESEARCH DESIGN AND METHODS

Animals

NOD mice, originally obtained from Professor Wu (Department of Endocrinology, Peking Union Medical College Hospital, Beijing, China), were housed and bred in animal facility of the KU Leuven since 1989. Housing of NOD mice occurred under semi-barrier conditions, and animals were fed sterile food and water *ad libitum*. NOD mice were screened for the onset of diabetes by evaluating glucose levels in urine (Clinistix; Bayer Diagnostics, Tarrytown, NY) and venous blood (Accu-Chek® Aviva, Roche Diagnostics Belgium, Vilvoorde, Belgium). Mice were diagnosed as diabetic when having positive glycosuria and two consecutive blood glucose measurements above 200 mg/dL. At the time of the experiments, the breeding stock had a diabetes incidence of 84% in female and 38% in male mice. Animals were maintained in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Experimental design

NOD mice were randomly assigned to be fed either a vitamin D₃-sufficient control diet or a vitamin D₃-supplemented diet during three periods of life. The control diet consisted of Ssniff® R/MNH diet plus 1% calcium, 0.7% phosphorus and 1,000 IU vitamin D₃/kg diet (4 IU a day/based on a consumption of 4 g of chow per 20 g of BW daily). The vitamin D₃-supplemented diet consisted of Ssniff® R/M-H diet plus 1% calcium, 0.7% phosphorus and 200,000 IU vitamin D₃/kg diet (800 IU a day) (Ssniff®, BioServices BV, Uden, The Netherlands). The different feeding periods were early-life (age 3–14 weeks), lifelong (age 3–35 weeks), and during pregnancy and neonatal life (until the end of lactation). All mice were monitored until 35 weeks of age. The research protocol of this study was approved and performed in accordance with the Ethics Committees of the KU Leuven (Leuven, Belgium).
Calcium and bone parameters

At 35 weeks of age, blood was collected by heart puncture and a femur was removed. Serum and femur were stored at -20°C until biochemical determinations were performed. Calcium content of the femur, measured on HCl dissolved bone ashes, and of the serum was analyzed by SYNCHRON Clinical Systems (Beckman Coulter, Analis SA, Suarlée, Belgium) and corrected for dry weight (bone). Phosphate levels in the serum were analyzed by the same system. Levels of osteocalcin and 25(OH)D$_3$ were measured using an in-house (15) and 25(OH)D$_3$ (DiaSorin NV, Anderlecht, Belgium) RIA kit, respectively.

Micro-computed tomography

Micro-computed tomography (µCT) analysis of the femur was performed ex vivo using the high resolution SkyScan 1172 system (settings: 50 kV, 200 µA, 0.5-mm Al filter, 5-µm pixel size; Bruker-microCT, Kontich, Belgium) as previously described (16). In brief, serial tomographs, reconstructed from raw data using the cone-beam reconstruction software (NRecon, v.1.4.4.0; Bruker-microCT), were used to calculate trabecular and cortical parameters, respectively from the metaphyseal and mid-diaphyseal area. A bone standard was used for the calibration of bone density measurements. Measurements were performed in laboratory of Clinical and Experimental Endocrinology, KU Leuven, Belgium, according to the guidelines of the American Society for Bone and Mineral Research.

Glucose tolerance test

Glycemia was measured in tail vein blood using a glucometer before and after (5, 15, 30, 60 and 120 min) an intra-peritoneal injection of 2 g D-glucose monohydrate per kg body weight, dissolved in sterile PBS.
**Histology, immunohistochemistry and insulin determination**

Severity of insulitis was assessed by histological screening of pancreatic sections of at least four animals per group imbedded in paraffin taken from experimental NOD mice at 35 weeks of age. Six µm tissue sections from formalin-fixed paraffin-embedded pancreases of each animal were cut and collected 100 µm apart, then stained with hematoxylin eosin. Islets were observed under light microscopy at 20× or 40×, enumerated and graded by an independent investigator in blinded fashion. At least 25 islets per pancreatic sample were scored for islet infiltration as follows: 0, no infiltration; 1, peri-insulitis; 2, lymphocyte infiltration in less than 50% of the islet; 3, lymphocyte infiltration in more than 50% of the islet; or 4, completely destroyed islets.

Immunofluorescence detection of FoxP3 expressing cells was performed on 6 µm thick paraffin tissue sections. Briefly, after antigen retrieval (10 mM citrate buffer pH=6, 10 min), sections were incubated with FITC-conjugated anti-Foxp3 primary antibody (5 µg/mL, clone FJK-16a, eBioscience) in TBS/proteinase I with 5% BSA (overnight, 4°C), followed by AlexaFluor (AF) 488-conjugated rabbit anti-FITC antibody (12.5 µg/mL, Molecular Probes, Invitrogen). For signal amplification, AF488-conjugated anti-rabbit IgG was used (8 µg/mL, Molecular Probes). Next, insulin was revealed using guinea pig anti-swine Ab (0.3 mg/mL, 60 min, DakoCytomation) and AF555-conjugated goat anti-guinea pig IgG (4 µg/mL, 60 min, Molecular Probes). The other half of each pancreas was used for insulin content determination as described (17).

**Flow cytometric analysis**

Single cell suspensions of spleen and pancreatic lymph nodes (PLN) were prepared from mice at 35 weeks of age. The following antibodies were used for staining: CD4 (GK1.5), CD8 (53-6.7), CD25 (PC61.5), CD11b (M1/70), CD11c (N418), I-A^k^ (10-3.6, cross-reactive with I-A^g7^ of NOD mice), the programmed death 1 ligand (PD-L1, MIH5), interferon-γ (IFNγ), FoxP3 and matching isotype
controls (all eBioscience). Cells were analyzed in a Gallios flow cytometer with Kaluza software (Beckman Coulter).

**Statistical Analysis**

Graphs were plotted and statistics calculated with GraphPad Prism (La Jolla, San Diego, CA). Differences in the incidence of diabetes were assessed using the Mantel-Cox Log-rank test. Results were expressed as mean ± the standard error of the mean (SEM) if normally distributed or as median and interquartile range if not normally distributed, and student’s t-test or Mann-Whitney test were used for comparison between groups, respectively. *P* values less than 0.05 were considered statistically significant (ns: not significant, *P* < 0.05, **P** < 0.01, ***P** < 0.001).
RESULTS

Safety of vitamin D₃ supplementation

Based on dose-titration studies (data not shown), we selected 800 IU per day (40,000 IU/kg BW/day) as a dose raising the vitamin D levels into the ‘highly vitamin D-sufficient’ zone (25(OH)D₃ concentration >230 nmol/L) without inducing hypercalcemia or bone decalcification. Only in mice that were treated lifelong (from 3 until 35 weeks of age), serum 25(OH)D₃ concentration increased to a mean concentration of 290 nmol/L (236 - 351 nmol/L) in male and female NOD mice at 35 weeks of age in comparison to 173 nmol/L (125 - 224 nmol/L) in the group on control diet (Table 1). This protocol did not perturb normal weight evolutions and did not cause significant alterations in serum phosphate or calcium levels (Table 1). On the other hand, mice supplemented with vitamin D₃ during pregnancy and lactation or during early-life (from 3 until 14 weeks of age) had 25(OH)D₃ concentrations comparable to mice fed control diet until 35 weeks of age (Supplementary Fig. 1).

Histological analysis of kidney and heart of the vitamin D₃ group revealed no calcifications in aorta and renal arteries (data not shown). To verify whether vitamin D₃ supplementation affected bone metabolism, serum osteocalcin and bone parameters were evaluated. Serum osteocalcin concentrations were not altered by lifelong vitamin D₃ supplementation (Table 1), indicating no alterations in bone turnover. Moreover, bone dry weight and ratio of calcium/bone dry weight was similar to control in the vitamin D₃ group at the end of the observation period (Table 1). The bone microstructure analysis by µCT showed that dietary vitamin D₃ did not affect bone mineral density and did not impair cortical or trabecular bone architecture (Fig. 1A and B). The vitamin D₃-supplemented diet did not affect any of the analyzed bone parameters (Fig. 1B). Overall, long-term vitamin D₃ supplementation was tolerated well and we found no indications of vitamin D toxicity and adverse metabolic side-effects.
**Vitamin D₃ supplementation reduces diabetes incidence in NOD mice when given early and lifelong**

We next assessed the effect of vitamin D₃ on diabetes development in the NOD mouse. Vitamin D₃ was given prophylactically at 800 IU per day during pregnancy and lactation (until 3 weeks of age), during early-life (from 3 until 14 weeks of age), or lifelong from early-life onwards (from 3 until 35 weeks of age). Only lifelong vitamin D₃ supplementation could significantly inhibit diabetes development in female and male NOD mice: 52% reduction in females and 58% reduction in diabetes incidence in males, as compared with mice on control diet (Fig. 2A). Only the long-term supplementation was effective in controlling diabetes progression, because shortening the period of vitamin D₃ treatment from 3 until 14 weeks of age caused a loss of diabetes protection by vitamin D₃: in females, the diabetes incidence in vitamin D₃-supplemented mice was comparable to the control group (Fig. 2B), whereas in males vitamin D₃ supplementation demonstrated a trend to decreased diabetes development, yet this was not significant (Fig. 2B). When given during pregnancy and lactation, vitamin D₃ supplementation had no effect on diabetes development either in female or male offspring (Fig. 2C).

**Lifelong vitamin D₃ supplementation preserves beta-cell function and reduces severe insulitis**

Prevention of diabetes by vitamin D₃ suggests preservation of sufficient beta-cell mass and/or function. To test this, we first did an intraperitoneal glucose tolerance test and found that the disease-free vitamin D₃ group controlled a glucose bolus with similar efficiency as normoglycemic NOD mice on control diet (Fig. 3A). Second, the insulin content of pancreases harvested from disease-free vitamin D₃-supplemented mice was comparable to amounts present in normoglycemic controls. Importantly, pancreases of disease-free vitamin D₃-supplemented animals contained significantly more insulin than pancreases of diabetic NOD mice on control diet at 35 weeks of age (Fig. 3B). Third, scoring of the insulitis revealed that immune infiltration in islets worsened in the control
group, whereas less severe insulitis, including a higher number of insulitis free islets, were observed in the majority of vitamin D₃-supplemented mice (Fig. 3C and D). Taken together, treatment efficiency was not only reflected in parameters measuring beta-cell function and mass, but also in the grade of insulitis.

**Vitamin D₃ supplementation decreases effector CD8⁺IFNγ and CD4⁺ IFNγ T-cells and increases CD4⁺(CD25⁺)FoxP3⁺ regulatory T-cells.**

We found that lifelong vitamin D₃ supplementation (from 3 until 35 weeks of age) did not cause immune depletion as the total number of lymphocytes in the spleen and PLN was similar to values found in sex-matched NOD mice on control diet (Fig. 4). Also, there were no significant differences in the frequencies of dendritic cell and macrophage subsets in spleen and PLN between the vitamin D₃ and control group (data not shown). Likewise, expression of the antigen-presenting molecule MHC class II I-A² and the programmed death 1 ligand (PD-L1) on antigen presenting cells was unaltered in mice receiving dietary vitamin D₃ supplementation (data not shown).

We did not observe differences in the absolute number and proportions of CD4⁺ and CD8⁺ T-cells in spleen and PLN after vitamin D₃ supplementation (Fig. 4 and Supplementary Fig. 2). However, lifelong vitamin D₃ supplementation induced a shift in the balance between effector T-cells (Teff) and regulatory T-cells (Tregs). As such, vitamin D₃ supplementation significantly decreased the frequency as well as the total number of CD8⁺ T-cells producing IFNγ in both spleen and PLN of female and male NOD mice (Fig. 5A and Supplementary Fig. 3). Moreover, the proportions and the absolute numbers of CD4⁺ T-cells expressing IFNγ were lower in the spleen of vitamin D₃-supplemented female mice, and in the PLN of vitamin D₃-supplemented males, in comparison to sex-matched NOD mice on control diet (Fig. 5B and Supplementary Fig. 3).

Expression of FoxP3, a transcription factor associated with Treg differentiation and function (18), is presently the most reliable marker to identify Tregs in mice. Female and male NOD mice receiving
lifelong vitamin D$_3$ supplementation showed a significant higher frequency of CD4$^+$FoxP3$^+$ Tregs in the PLN but not in spleen, as compared to sex-matched mice on control diet (Fig. 6A). Analysis of CD25 expression on CD4$^+$FoxP3$^+$ T-cells revealed that this increase concerned activated Tregs, which were increased not only in spleen but also in the PLN of vitamin D$_3$-treated mice (Fig. 6B) (19,20). Of note, vitamin D$_3$ supplementation during pregnancy and lactation or during early-life (from 3 until 14 weeks of age) did not increase the percentages of CD4$^+(CD25^+)FoxP3^+$ T-cells in spleen or PLN (Supplementary Fig. 4).

Enumeration of FoxP3$^+$ cells in the pancreas revealed higher numbers of FoxP3$^+$ T-cells in the islet infiltrates of vitamin D$_3$-supplemented mice, especially in the females, compared to diabetic controls, (Fig. 7), suggesting these Tregs suppress autoreactive responses not only in the PLN, but also at the site of inflammation.
DISCUSSION

Vitamin D is a pro-hormone that is well-known for its role in calcium homeostasis and bone mineralization and metabolism, but accumulating data suggest additional roles in many important body functions, including the regulation of both innate and adaptive immune responses. In humans, the daily vitamin D intake recommended by the Institute of Medicine (IOM) is 400 IU per day for children less than 1 year, up to 600 IU per day for all individuals between 1 and 70 years, assuming already some sunlight exposure, and 800 IU per day for individuals older than 70 years (21). However, many clinicians suggest that, for a good health state and to allow extra-skeletal benefits, the daily supply of vitamin D from all sources should be greater than 800 IU.

Until now, data are very limited on whether dietary vitamin D supplements can in fact modify the course of autoimmune diseases such as type 1 diabetes in humans. These studies often lack precise information on dosage and regimen of vitamin D supplementation, which can lead to contradictory results. Meta-analysis of records from 4 case-control studies and one cohort study imply that the risk of type 1 diabetes development significantly reduced in children treated with regular vitamin D between 7 and 12 months of age compared to those who were supplemented before 7 months of age or not supplemented at all (5,22). A recent meta-analysis of 2 cohort studies and 6 case-control studies on vitamin D intake during early-life corroborated these findings (23). The importance of maternal vitamin D intake during pregnancy is also not clear: a case-control study in Norway indicated that the risk of type 1 diabetes was higher in the offspring of women with the lowest 25(OH)D concentrations (10), whereas other studies did not find any correlation between maternal serum 25(OH)D values and type 1 diabetes risk in the offspring (24,25). Only properly-designed, randomized and long-term trials will bring the definitive answer.

Here, we provide the first evidence that only early and lifelong vitamin D₃ supplementation with 800 IU per day, which significantly increased serum 25(OH)D₃ levels above sufficiency and corrected the immunological defects in diabetes-prone mice that already had sufficient, even high, vitamin D₃
levels, safely reduced diabetes development. Vitamin D₃ supplementation prevented severe insulitis and preserved beta-cell mass best. In contrast, a short and early intervention (age 3–14 weeks) with regular vitamin D₃ was less protective, in line with our previous study showing that short-term i.p. administration of 1,000 IU of vitamin D₃ to NOD mice early in life, corresponding to neonatal life and childhood in man, was insufficient to protect against diabetes (13). Of note, we did not evaluate the effects of vitamin D supplementation at a later stage in life (e.g. age 14–35 weeks), as we demonstrated previously that a late intervention with the bioactive vitamin D₃ metabolite (from 14 weeks of age, when insulitis is present) failed to prevent disease (26).

Administration of vitamin D via the oral route may favor regulation of tight junction proteins necessary to maintain mucosal integrity (27). Moreover, Ooi et al. demonstrated that oral bioactive vitamin D₃ can regulate the composition of the gut bacterial microflora and that deficiency of vitamin D or its receptor (VDR) can cause impaired epithelial integrity, dysbiosis, increased inflammation, and more severe experimental colitis (28). Active vitamin D also regulates the development and function of regulatory iNKT-cells and intraepithelial CD8αα lymphocytes in the gut (28,29). These data imply that vitamin D supplementation might be a way to modulate the gut microflora and its microbiome and consequently regulates gastrointestinal innate and adaptive immune responses.

Recently, Roy et al. suggested that not just the antagonistic interaction between Treg and Teff cells, but also their continuous regulation by vitamin D ultimately determines the outcome of an (auto)immune disease (30). This implies that optimal vitamin D concentrations are vital in keeping the balance between Treg and Teff cells in individuals (or animals) with an underlying immune dysfunction. In fact, female and male NOD mice on a control (vitamin D₃-sufficient) diet have normal concentrations of serum 25(OH)D₃ (mean of 173 nmol/L) and still present a high incidence of diabetes. Thus, our data suggest that, in NOD mice, 25(OH)D₃ concentrations of more than 230 nmol/L may be required to modulate immune dysregulation. Although further studies are needed, it
should be noted that a recent report from the IOM indicated that serum 25(OH)D$_3$ concentrations in humans above 125 nmol/L may result in potential adverse side reactions and there should be caution against overtreatment (21).

Several studies in human subjects evaluated the influence of administering vitamin D to pregnant and/or breastfeeding women on the risk of developing type 1 diabetes in their children (31,32). Despite some early studies suggesting a protective effect of vitamin D, a meta-analysis by Zipitis and Akobeng concluded that supplementing pregnant women with vitamin D had no implications on type 1 diabetes development in their infants (22). Our current observations in NOD mice are consistent with this. Moreover, our data showing that vitamin D$_3$ supplementation during lactation failed to prevent disease in NOD mice are in line with an earlier study showing that 16 IU of vitamin D per day from conception until 10 weeks of age does not suffice to interfere with disease progression in NOD mice (33). It is suggested that during this early period the adaptive immune system has not yet fully matured and thus escapes the immunomodulatory action of vitamin D. As such, supplementation with vitamin D$_3$ during pregnancy and lactation in already vitamin D$_3$-sufficient mothers will not have immunoregulatory actions on the hyperactivity of effector immune cells against self-antigens. Third, we found that our vitamin D$_3$ protocol given from early-life on and lifelong did not cause immune depletion, as total number of lymphocytes and T-cell subsets (e.g. CD4, CD8) in spleen and PLN remained unchanged. On the other hand, vitamin D$_3$ supplementation dampened Th1 responses, which are often associated with autoimmune responses (34), and skewed the Teff/Treg balance in favor of Tregs in diabetes-prone mice. Interestingly, the increase of Tregs concerned activated Tregs according to CD25 expression (20,35). Our observations are supported by several in vitro and in vivo findings. The active form of vitamin D exerts a marked inhibitory effect on the adaptive immune cells by inhibiting Th1 polarization (36) and triggering the emergence of Tregs with the functional capacity to suppress activation and proliferation of Teff cells (37,38). Here, we report that upon vitamin D$_3$ supplementation these Treg (subsets) preferentially accumulate in
draining PLN and pancreatic islets. Our group had already documented that a vitamin D analog had the capacity to imprint human T-cells with a specific homing signature favoring migration to sites of inflammation (37).

Finally, caution is warranted when interpreting these data, because not only lifelong administration of vitamin D$_3$ was necessary for protection, but extremely high doses were needed, namely more than 200 times the amount of vitamin D normally present in the daily amount of food of a mouse. Converting these doses to humans is difficult and we advocate that rather the serum concentrations of 25(OH)D$_3$ reached after supplementation versus baseline in the mice should be considered. The values achieved in this study (mean of 290 nmol/L) lie well above the present advocated targets of 50-100 nmol/L for humans (39), but it is important to keep in mind that control NOD mice are already very vitamin D-sufficient (serum 25(OH)D$_3$ concentrations >150 nmol/L). If we apply the estimate of Heaney et al. (40) that a 40 IU per day increment in intake raises 25(OH)D$_3$ by 0.7 nmol/L in healthy men with a mean baseline value of 70 nmol/L, then a dose of 12,500 IU or more vitamin D$_3$ per day will be needed to raise serum 25(OH)D$_3$ concentrations to 290 nmol/L. These doses are thus completely out of the range of the suggested supplements for bone health (39), and may lead to calcemic side-effects when administered long-term. At present, it is thus not advised to systematically administer these high doses in humans, as randomized intervention trials will be needed to demonstrate or refute the potential of these mega doses in diabetes prevention in humans.

In conclusion, lifelong dietary supplementation with high doses of regular vitamin D$_3$ brings 25(OH)D$_3$ concentrations above 230 nmol/L, prevents diabetes in NOD mice in a safe way and is accompanied by induction of Tregs. This preclinical study confirms the potential of exploiting the vitamin D system in the prevention of type 1 diabetes in humans, but the fact that only lifelong supplementation with high doses altered disease presentation warns for overly optimistic statements on duration of treatment.
CONTRIBUTION STATEMENT

TT, LD, FB, IS, GS, and JL acquired data. TT, GC, AV, FD, CG and CM interpreted the data. TT, AV, GC, FD, TVB, CG and CM drafted the article. TVB, CG and CM revised the manuscript critically for important intellectual content. All authors gave final approval of the version to be published.

ACKNOWLEDGEMENTS

We thank Erik Van Herck, Ivo Jans, Suzanne Marcelis, Jaak Billen, Karolien Ciotkowski and Martine Gillis (CEE, KU LEUVEN, Belgium) for excellent technical assistance. Also Riet Van Looveren, Karen Moermans, Ingrid Stockmans, Nick van Gastel and Steve Stegen (CEE, KU LEUVEN, Belgium) for the assistance with the µCT analysis. This work was supported by grants from the Fund for Scientific Research Flanders (FWO-Vlaanderen G.0734.10), the University of Leuven (KU LEUVEN GOA 2009/10 and 2014/10), the European Community's Health Seventh Framework Programme (FP7/2009-2014 under grant agreement 241447 with acronym NAIMIT). LD is supported through an IRO fellowship of the KU Leuven. TT is supported through a PDM postdoctoral fellowship of the KU LEUVEN. CM is a clinical researcher of the FWO-Vlaanderen. CG is supported by the KU LEUVEN. FD received support from the Italian Ministry of Research, the Italian Ministry of Health, and the Tuscany Region. TT, CM and CG are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

The authors declare that there is no duality of interest associated with this manuscript.
REFERENCES


29. Ooi JH, Chen J, Cantorna MT: Vitamin D regulation of immune function in the gut: why do T-cells have vitamin D receptors? Molecular aspects of medicine 2012;33:77-82


TABLE LEGENDS

Table 1. Vitamin D and calcium metabolism after vitamin D₃ supplementation in NOD mice.
Summary data (mean ± SEM) from female and male (n = 4–5/experiment). NOD mice were on normal chow (4 IU vitamin D, control) or chow supplemented with 800 IU per day of vitamin D₃. Statistical significance was calculated by student t-test. *** P < 0.001 vs. controls.
Table 1: Vitamin D and calcium metabolism after vitamin D<sub>3</sub> supplementation in NOD mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>controls</td>
<td>800 IU</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>26.0±0.7</td>
<td>32.2±0.4</td>
</tr>
<tr>
<td>Serum 25(OH)D&lt;sub&gt;3&lt;/sub&gt; (nmol/L)</td>
<td>301.3±20.9***</td>
<td>160.3±5.1</td>
</tr>
<tr>
<td>Serum calcium (mmol/L)</td>
<td>2.4±0.1</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>Serum phosphate (mmol/L)</td>
<td>3.2±0.3</td>
<td>3.7±0.3</td>
</tr>
<tr>
<td>Serum osteocalcin (ng/mL)</td>
<td>52.2±5.8</td>
<td>49.5±5.8</td>
</tr>
<tr>
<td>Bone dry weight (mg)</td>
<td>51.1±0.9</td>
<td>52.7±0.7</td>
</tr>
<tr>
<td>Calcium/bone dry weight</td>
<td>0.30±0.21</td>
<td>0.36±0.21</td>
</tr>
</tbody>
</table>

*** P < 0.001 vs. controls.
FIG. LEGENDS

Fig. 1. Dietary vitamin D₃ supplementation does not affect bone parameters. A: µCT-based visualization of trabecular bone in femur from control (upper panels) and vitamin D₃-supplemented NOD mice (lower panels). B: From left to right: quantification of the trabecular bone volume (BV/TD), trabecular thickness (Tb.Th), trabecular number (Tb.N) and cortical thickness (Ct.Th) in control mice (white symbols) and long-term 800 IU vitamin D₃-supplemented mice (grey symbols) at 35 weeks of age. Statistical significance was calculated using Student t-test. ns: not significant.

Fig. 2. Lifelong vitamin D₃ supplementation reduces diabetes incidence in NOD mice. Female (circles) and male (diamonds) NOD mice were given control chow (white symbols) until 35 weeks of age, or given chow supplemented with 800 IU of vitamin D₃ per day (grey symbols) between 3 until 35 weeks of age (lifelong) (A), between 3 until 14 weeks of age (early-life) (B), during coupling until 3 weeks of age (pregnancy and lactation) (C). Dietary regimens are depicted graphically; in grey is the period when mice received the vitamin D₃-supplemented diet. Mice with two consecutive measurements of blood glucose levels >200 mg/dL were considered diabetic. Statistical significance was calculated versus NOD mice on control chow by Mantel-Cox log-rank test. * P <0.05, ** P <0.01, *** P < 0.001.

Fig. 3. Lifelong vitamin D₃ supplementation preserves insulin content and decreases severe insulitis. (A) Intraperitoneal glucose tolerance tests (IPGTT) were performed at the end of the observation period (at 35 weeks of age) on experimental groups, as indicated. (B) Evaluation of insulin content in pancreatic extracts from experimental groups by ELISA, as indicated. Data are expressed as ng/mg of pancreas. Graphs show median and interquartile range. Statistical significance between two groups was calculated using Mann-Whitney test. ns: not significant; * P <0.05, ** P <0.01, *** P <
Pancreatic sections of indicated groups were stained with H&E and insulitis was scored as indicated in Research design and methods, from left to right graphs showing % of insulitis, % of insulitis free islets and % of islets with heavy insulitis in vitamin D supplemented mice vs. control normoglycemic and diabetic mice. (D) For the evaluation of insulitis the following score was used: 0 (white - intact islets), 1 (vertical lines - peri-insulitis), 2 (grey - <50% infiltration), 3 (horizontal lines - >50% infiltration), IV (black - complete destruction). Statistical significance between two groups was calculated using Mann-Whitney test; ns: not significant; * P < 0.05, ** P < 0.01, *** P < 0.001.

Fig. 4. Unaltered lymphocyte number and subsets in spleen and PLN of mice following lifelong vitamin D$_3$ supplementation. Spleen and PLN were harvested from NOD mice (n = 5–7) at 35 weeks of age. The absolute number of lymphocytes (A), CD4$^+$ (B) and CD8$^+$ T-cell subsets (C) were measured by flow cytometry in the spleen (left panels) and PLN (right panels) of control (white symbols) and vitamin D$_3$-supplemented mice (grey symbols). Graphs show median and interquartile range. Statistical significance was calculated by Mann-Whitney test.

Fig. 5. Decrease of CD8$^+$IFN$\gamma^+$ T-cells in spleen and PLN after lifelong vitamin D$_3$ supplementation. Spleen and PLN were harvested from NOD mice (n = 5–7) at 35 weeks of age. The frequencies of CD8$^+$IFN$\gamma^+$ (A) and CD4$^+$IFN$\gamma^+$ (B) T-cell subsets were measured by flow cytometry and displayed as percentage of IFN$\gamma^+$ in CD8$^+$ (A) or CD4$^+$ (B) gate in the spleen (left panels) and PLN (right panels) of control (white symbols) and vitamin D$_3$-supplemented mice (grey symbols). Graphs show median and interquartile range. Statistical significance was calculated by Mann-Whitney test, * P < 0.05.
Fig. 6. Lifelong vitamin D₃ supplementation increases Treg frequencies. At 35 weeks of age, spleen (right panels) and PLN (left panels) were harvested from NOD mice (n = 5–6) and the frequency of Tregs was determined as percentage of FoxP3⁺ in the CD4⁺ fraction (A) and activated Tregs as percentage of CD25⁺FoxP3⁺ in the CD4⁺ gate (B) using flow cytometry. Graphs show median and interquartile range. Statistical significance was calculated using Mann-Whitney test. * P < 0.05, ** P < 0.01.

Fig. 7. Increased numbers of FoxP3⁺ cells in the pancreatic infiltrates of lifelong vitamin D₃-supplemented mice. Number of FoxP3⁺expressing cells in pancreatic infiltrates as determined by manual counting of FoxP3⁺ cells on immunostained paraffin sections (n = 6–14). Scatter plots show median with interquartile range. Statistical significance was calculated using Mann-Whitney test, ns: not significant, * P < 0.05, ** P < 0.01, *** P < 0.001.

Supplementary Fig. 1. Serum 25(OH)D₃ concentrations were significantly increased following lifelong vitamin D₃ supplementation. At 35 weeks of age, serum was analyzed for 25(OH)D₃ metabolite in mice on vitamin D₃-supplemented and control diet. Supplemented mice received 800 IU per day of vitamin D₃ either during pregnancy and lactation, early life (from 3 until 14 weeks of age) or lifelong (from 3 until 35 weeks of age). Data are mean ± SEM. Statistical significance was calculated by student t-test. *** P < 0.001 vs. control.

Supplementary Fig. 2. Lifelong vitamin D₃ supplementation keeps CD4⁺ and CD8⁺ T-cell frequencies. At 35 weeks of age, spleen and PLN were isolated from experimental groups (n = 5–7), as indicated. Scatter plots representing frequency of CD4⁺ (A) and CD8⁺ T-cells (B) in spleen (left panels) and PLN (right panels) of mice fed vitamin D₃-supplemented or control diet. Graphs show
median and interquartile range. Statistical significance between two groups was calculated using Mann-Whitney test; * $P < 0.05$

Supplementary Fig. 3. Lifelong vitamin D$_3$ supplementation increases absolute number of IFN$\gamma^+$CD$8^+$ T-cells in PLN. At 35 weeks of age, spleen and PLN were isolated from experimental groups ($n = 5–7$). Scatter plots showing number of IFN$\gamma^+$CD$8^+$ (A) and IFN$\gamma^+$CD$4^+$ (B) T-cells in spleen (right panels) and PLN (left panels) of mice fed vitamin D$_3$-supplemented or control diet. Plots show median and interquartile range. Statistical significance was calculated using Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Supplementary Fig. 4. Vitamin D$_3$ supplementation increases Treg frequencies only when given lifelong. Supplemented mice received 800 IU vitamin D$_3$ per day either during pregnancy and lactation, early-life (from 3 until 14 weeks of age) or lifelong (from 3 until 35 weeks of age). At 35 weeks of age, spleen (right panels) and PLN (left panels) were harvested from NOD mice ($n = 5–6$) and the frequency of Tregs was determined as the percentage of FoxP3$^+$ in the CD$4^+$ T-cell fraction (A) and activated Tregs as percentage of CD25$^+$FoxP3$^+$ T-cells in the CD$4^+$ gate (B) using flow cytometry. Graphs show median and interquartile range. Statistical significance was calculated using Mann-Whitney test. * $P < 0.05$, ** $P < 0.01$. 
Figure 1

A

female
male
control
800 IU vitD

B

<table>
<thead>
<tr>
<th></th>
<th>BV/TV (%)</th>
<th>Tb.Th (µm)</th>
<th>Tb.N (1/mm)</th>
<th>Ct.Th (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>800 IU vitD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ns
**Figure 2**

### Females
- Control
- Vitamin D 800 IU

### Males
- Control
- Vitamin D 800 IU

**A**
- Control diet (4 IU) vs. Vitamin D, 800 IU
- Coupling, birth, 3 wks (weaning), 14 wks, 35 wks

**B**
- Control diet (4 IU) vs. Vitamin D, 800 IU
- Control diet (4 IU)

**C**
- Vitamin D, 800 IU vs. Control diet (4 IU)

Diabetic mice (%) vs. Age (weeks)
Figure 3

A

IPGTT (glycemia mg/dL)

(min post glucose challenge)

females

- diabetic control n=2
- normoglycemic control n=5
- vit D 800 IU n=4

males

- diabetic control n=3
- normoglycemic control n=4

B

Insulin content (ng/mg of pancreas)

females

- normoglycemic
diabetic
vit D 800 IU

males

- normoglycemic
diabetic
vit D 800 IU

C

% insulitis-free per mouse

% heavy insulitis per mouse

D

Score 0 no insulitis
Score I Peri-insulitis
Score II <50% infiltration
Score II >50% infiltration
Score IV complete destruction

Score 0 no insulitis
Score I Peri-insulitis
Score II <50% infiltration
Score II >50% infiltration
Score IV complete destruction

ns ns

ns ns

ns ns

ns ns

** **

** **

** **

** **

ns ns

ns ns

ns ns

E

ns ns

ns ns

ns ns

ns ns
Figure 5

A

**spleen**

<table>
<thead>
<tr>
<th></th>
<th>females</th>
<th>males</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td><img src="chart1.png" alt="" /></td>
<td><img src="chart2.png" alt="" /></td>
</tr>
<tr>
<td>800 IU</td>
<td><img src="chart3.png" alt="" /></td>
<td><img src="chart4.png" alt="" /></td>
</tr>
<tr>
<td>VitD</td>
<td><img src="chart5.png" alt="" /></td>
<td><img src="chart6.png" alt="" /></td>
</tr>
</tbody>
</table>

* p = 0.08

B

<table>
<thead>
<tr>
<th></th>
<th>females</th>
<th>males</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td><img src="chart7.png" alt="" /></td>
<td><img src="chart8.png" alt="" /></td>
</tr>
<tr>
<td>800 IU</td>
<td><img src="chart9.png" alt="" /></td>
<td><img src="chart10.png" alt="" /></td>
</tr>
<tr>
<td>VitD</td>
<td><img src="chart11.png" alt="" /></td>
<td><img src="chart12.png" alt="" /></td>
</tr>
</tbody>
</table>

* p = 0.05

Diabetes
Figure 6

**Diabetes**

### Spleen

<table>
<thead>
<tr>
<th>Gender</th>
<th>Treatment</th>
<th>% FoxP3+ in CD4+ gate</th>
<th>% CD25FoxP3+ in CD4+ gate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>control</td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
<tr>
<td>Females</td>
<td>800 IU vitD</td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
<tr>
<td>Males</td>
<td>control</td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
<tr>
<td>Males</td>
<td>800 IU vitD</td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
</tbody>
</table>

### PLN

<table>
<thead>
<tr>
<th>Gender</th>
<th>Treatment</th>
<th>% FoxP3+ in CD4+ gate</th>
<th>% CD25FoxP3+ in CD4+ gate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>control</td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
<tr>
<td>Females</td>
<td>800 IU vitD</td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
<tr>
<td>Males</td>
<td>control</td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
<tr>
<td>Males</td>
<td>800 IU vitD</td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
</tbody>
</table>
Supplementary Figure 2

**spleen**

A

<table>
<thead>
<tr>
<th></th>
<th>females</th>
<th>males</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td><img src="image1" alt="Graph" /></td>
<td><img src="image2" alt="Graph" /></td>
</tr>
<tr>
<td>800 IU vitD</td>
<td><img src="image3" alt="Graph" /></td>
<td><img src="image4" alt="Graph" /></td>
</tr>
</tbody>
</table>

**PLN**

B

<table>
<thead>
<tr>
<th></th>
<th>females</th>
<th>males</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td><img src="image5" alt="Graph" /></td>
<td><img src="image6" alt="Graph" /></td>
</tr>
<tr>
<td>800 IU vitD</td>
<td><img src="image7" alt="Graph" /></td>
<td><img src="image8" alt="Graph" /></td>
</tr>
</tbody>
</table>

- 

Diabetes
Supplementary Figure 3

A

**spleen**

<table>
<thead>
<tr>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>800 IU vitD</td>
</tr>
<tr>
<td>control</td>
<td>800 IU vitD</td>
</tr>
</tbody>
</table>

B

**PLN**

<table>
<thead>
<tr>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>800 IU vitD</td>
</tr>
<tr>
<td>control</td>
<td>800 IU vitD</td>
</tr>
</tbody>
</table>