

The Vitamin D Receptor in Thyroid Development and Function

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Key Words

Vitamin D receptor · Thyroid · Calcitonin · Vitamin D · Calcium

Abstract

Background and Objective: Vitamin D is known to modulate thyroid neoplastic and autoimmune disease. We investigated the role of the vitamin D receptor (VDR) in normal thyroid development and function (thyrocytes and C cells). **Methods:** The thyroid phenotype of VDR knockout mice was studied in comparison to wild-type controls. The mice were fed a normal diet or a calcium-rich diet to circumvent effects induced by hypocalcemia. **Results:** Thyroid morphology was unaltered in VDR knockout mice. Also, expression of different parameters of thyrocyte function was comparable (immunohistochemistry). C cell physiology was, however, affected in the absence of the VDR, resulting in increased thyrocyte calcitonin expression (immunohistochemistry), paralleled by increased serum calcitonin levels, but only in normocalcemic mice. To study a possible effect of vitamin D status on basal calcitonin levels in humans, serum calcitonin concentrations were compared between vitamin D-deficient and -sufficient patients (serum 25-OH vitamin D₃ ≤10

and ≥40 ng/ml, respectively), but no difference was observed. **Conclusions:** In mice, the VDR is redundant for normal thyrocyte function, but not for C cell function, where it mediates the negative control of calcitonin by 1,25-dihydroxyvitamin D₃. In patients, vitamin D status does not affect basal serum calcitonin levels. A study in healthy individuals is needed to confirm these findings.

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Introduction

1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the active form of vitamin D, is mainly known for its effects on calcium and phosphate homeostasis with bone, intestine and kidney as principal target tissues. However, 1,25(OH)₂D₃ has pleiotropic effects which include anti-proliferative, anti-inflammatory and prodifferentiating effects [1]. Several lines of evidence suggest a role for vitamin D in thyroid disease. The ligand for 1,25(OH)₂D₃, the nuclear vitamin D receptor (VDR), is expressed in many tissues including benign and malignant thyroid tissue [2, 3].

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1,25(OH)₂D₃ concentration dependently inhibits TSH-stimulated iodide uptake in rat thyroid follicular (FRTL-5) cells [4]. We and others have shown that high doses of 1,25(OH)₂D₃ or structural analogues have anti-proliferative effects in human thyroid cancer cell lines [5–7]. On the other hand, data on a possible association between vitamin D deficiency and increased risk for thyroid cancer are scarce and debated [8–10]. Several studies also suggest a link between vitamin D and autoimmune thyroid disease. 1,25(OH)₂D₃ protects human thyrocytes from programmed cell death via increased Bcl-2 expression and prevents autoimmune thyroiditis in mice [11, 12]. The immunomodulatory effect of 1,25(OH)₂D₃ or the analogue elocalcitol is exerted through decreased expression of HLA class II molecules on thyrocytes and impaired Th1-mediated inflammatory responses in thyrocytes, while the T cell response is shifted towards a Th2 phenotype [13, 14]. Furthermore, polymorphisms of VDR and CYP27B1 – the enzyme responsible for the activation of the vitamin D precursor 25-hydroxyvitamin D₃ (25-OHD₃) into active 1,25(OH)₂D₃ – have been associated with increased risk for autoimmune thyroid disease and thyroid cancer in some studies [15, 16], although other studies found no association [17, 18]. Finally, vitamin D deficiency is reported to correlate with the presence of antithyroid antibodies and abnormal thyroid function in humans [19, 20], and to modulate autoimmune hyperthyroidism in mice [21].

Apart from inhibiting osteoclast-mediated bone resorption and increasing renal calcium and phosphate excretion, calcitonin (produced by the parafollicular C cells) is known to be involved in the homeostasis of 1,25(OH)₂D₃. More specifically, together with parathormone (PTH), calcitonin is a positive regulator of CYP27B1. Whereas PTH is mainly active under hypocalcemic conditions, calcitonin is the main regulator under normocalcemic to hypercalcemic conditions [22, 23]. On the other hand, CYP27B1 is negatively regulated by fibroblast growth factor 23 (FGF23). PTH and FGF23 are known to be regulated by 1,25(OH)₂D₃ through the VDR [24]. High 1,25(OH)₂D₃ levels decrease calcitonin production in C cells in rats, suggesting that 1,25(OH)₂D₃ negatively controls calcitonin. However, the involvement of the VDR in this negative feedback has not yet been demonstrated [25, 26].

In human physiology and pathophysiology, the role of 1,25(OH)₂D₃-VDR signaling in the regulation of thyrocyte and C cell homeostasis is unclear, but as vitamin D deficiency and thyroid dysfunction are both very prevalent and as serum calcitonin is widely used as a specific

tumor marker in the workup of thyroid nodular disease and in the follow-up of patients with medullary thyroid cancer [27], further insight is needed in the role of 1,25(OH)₂D₃-VDR signaling in thyroid physiology.

In this study we used systemic VDR knockout (KO) mice to investigate the effect of impaired VDR signaling on thyroid development and function, studying both thyrocyte and C cell function. VDR-KO mice are characterized by hypocalcemia, secondary hyperparathyroidism and bone deformities. These effects can be prevented by a calcium-rich diet from weaning on [28].

Materials and Methods

Mice

Systemic Leuven VDR-KO mice were generated as described [28] and housed in our animal facility under conventional conditions. Wild-type (WT) sex-matched littermates were used as controls. The mice were kept on a normal diet (Ssniff R/M-H; Ssniff, Soest, Germany) or a calcium-rich diet (20% lactose/2% calcium/1.25% phosphorous, diet TD96348; Harlan, Horst, The Netherlands) in order to exclude effects induced by hypocalcemia. Food and water were given ad libitum. The diets were started immediately after weaning. Animals were sacrificed at 10–12 weeks of age and the entire thyroid as well as blood (heart puncture) were collected. The thyroid was embedded in OCT, snap-frozen in liquid nitrogen and stored at –80°C until use. Serum was stored at –20°C until use. All experimental procedures were approved by the Ethical Committee of the KU Leuven.

Histology and Histomorphometry

Mouse thyroid cryostat sections (7 μm) were mounted on glass slides followed by hematoxylin and eosin staining according to standard protocols. Morphometric analysis of the thyroid glands of VDR-WT and -KO mice on both diets (n = 3 mice per group) was performed using the point-counting method described by Weibel et al. [29]. One thousand points were counted for each thyroid, and the relative volumes of epithelium and follicular lumen (colloid) were calculated and expressed as percentage relative volume.

Immunohistochemistry

Iodinated thyroglobulin (Tg-I), thyroxin (T4), dual oxidase (Duox), calcitonin, caspase-6 and 4-hydroxynonenal (4-HNE) immunostaining were performed on frozen sections. With the exception of 4-HNE, sections were rehydrated with PBS-BSA (1%) and thereafter incubated in PBS-BSA (1%) containing 1:50 goat serum. 4-HNE sections were incubated with PBS-BSA (5%) containing 1:30 goat serum. The conditions for immunohistochemistry are summarized in table 1. All sections were incubated with the first antibody (Tg-I, T4, Duox, calcitonin, caspase-6 and 4-HNE) at room temperature. The binding of antibodies was detected using a second antibody conjugated to a peroxidase-labeled polymer (EnVision detection; DakoCytomation, Heverlee, Belgium). Peroxidase activity was revealed by AEC substrate (3-amino-9-ethylcarbazole, Dako). Finally, sections were counterstained

Table 1. Experimental conditions for immunohistochemistry

Antibody	1° Antibody
Tg-I (J.J.M. De Vijlder, Amsterdam, The Netherlands)	mouse monoclonal, 1:2,000, 1 h
T4 (MyBiosource, San Diego, Calif., USA)	mouse monoclonal, 1:800, overnight
Duox 1/2 (F. Miot, ULB, Brussels, Belgium)	rabbit polyclonal, 1:75, 4 h
Caspase-6 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA)	rabbit polyclonal antibody, 1:75, 1 h
4-HNE (Calbiochem, Darmstadt, Germany)	rabbit polyclonal, dilution 1:500, overnight
Calcitonin (Dako, Heverlee, Belgium)	rabbit polyclonal, 1:500, 3 h

with Mayer's hematoxylin, rinsed and mounted in Faramount Aqueous Mounting Medium (Dako). Each immunostaining was performed on thyroid sections of VDR-WT and -KO mice on both diets (n = 3–6 mice per group), and evaluated by two independent investigators (I.C. and A.G.). One representative experiment for each immunostaining is shown.

Mouse Serum Biochemistry

Total serum calcium and creatinine was analyzed by Synchron Clinical Systems (Beckman Coulter, Suarlée, Belgium). Serum calcitonin levels were measured with a two-site immunoradiometric assay (Immutopics, San Clemente, Calif., USA). Briefly, two antibodies namely ¹²⁵I-labeled calcitonin antibody and calcitonin antibody immobilized on plastic beads were added to the samples. After an 18-hour incubation period, the beads were washed three times and the counts were recorded in a gamma counter. Serum TSH was evaluated by bioassay using a line of Chinese hamster ovary cells (CHO-K1) stably transfected with human TSH receptor cDNA, as previously described [30]. Five to eleven animals per group were analyzed separately.

Patient Serum Biochemistry

Serum samples from patients were randomly selected in a 1:1 male:female ratio in our hospital laboratory department based on the presence of either low or high 25-OHD₃ levels (≤ 10 and ≥ 40 ng/ml, respectively), using an equilibrium radioimmunoassay (DiaSorin, Stillwater, Minn., USA). Intra- and interassay coefficients of variation were 11 and 9%, respectively. The detection limit of the radioimmunoassay kit was 1.5 ng/ml. For each patient the following data were retrieved from the medical chart: age, sex, previous or current thyroid or parathyroid disease, current use of proton pump inhibitor, serum calcium, renal function (serum creatinine and eGFR), and serum TSH level in the same serum sample as used for the 25-OHD₃ determination. Patients hospitalized at the intensive care unit, patients with known thyroid or parathyroid disease, or with neuroendocrine tumors were excluded for further analysis, as well as patients with severe hypocalcemia or hypercalcemia or with an abnormal serum TSH.

A total of 48 patient sera (4 groups, n = 12 patients per group) were thus collected and stored for additional calcitonin determination, using a radioimmunoassay (Biosource, Nijvel, Belgium). Intra- and interassay coefficients of variation were 1.4 and 4.6%, respectively. The detection limit of the radioimmunoassay kit was 0.9 pg/ml.

Statistical Analysis

Data are expressed as means \pm SEM. For comparisons of numerical data between VDR-WT and -KO mice, and between patients with low and high 25-OHD₃ levels, an unpaired Student's t test was used, unless otherwise stated. A two-sided p value of <0.05 was considered statistically significant.

Results

Disrupted VDR Signaling Does Not Affect Thyroid Morphology in Mice

We first investigated whether thyroid histology of 10- to 12-week-old VDR-KO mice was different from their WT littermates. On hematoxylin and eosin staining, no difference was observed in the thyroid follicles or thyrocytes between both groups fed a normal diet. As expected, serum calcium levels were significantly lower in VDR-KO mice compared to WT mice (7.5 ± 0.42 vs. 10.0 ± 0.2 mg/dl, respectively; $p < 0.0001$). Renal function (creatinine) was not different (data not shown). Since many of the observed effects in VDR-KO mice are secondary to their hypocalcemic status, we subsequently evaluated thyroid morphology in mice fed a calcium-rich diet, resulting in a correction of the hypocalcemia (9.6 ± 1.6 and 10.7 ± 0.9 mg/dl for VDR-WT and -KO mice, respectively). Also under normocalcemic conditions, we did not observe morphological differences between VDR-WT and -KO thyroid glands (fig. 1a). Furthermore, thyroid sections also showed no signs of immune infiltration or neoplasia in any of the groups. Further quantitative morphometric investigation using the point-counting method confirmed the absence of difference in thyrocyte size or colloid/thyrocyte ratio between VDR-WT and -KO mice (fig. 1b).

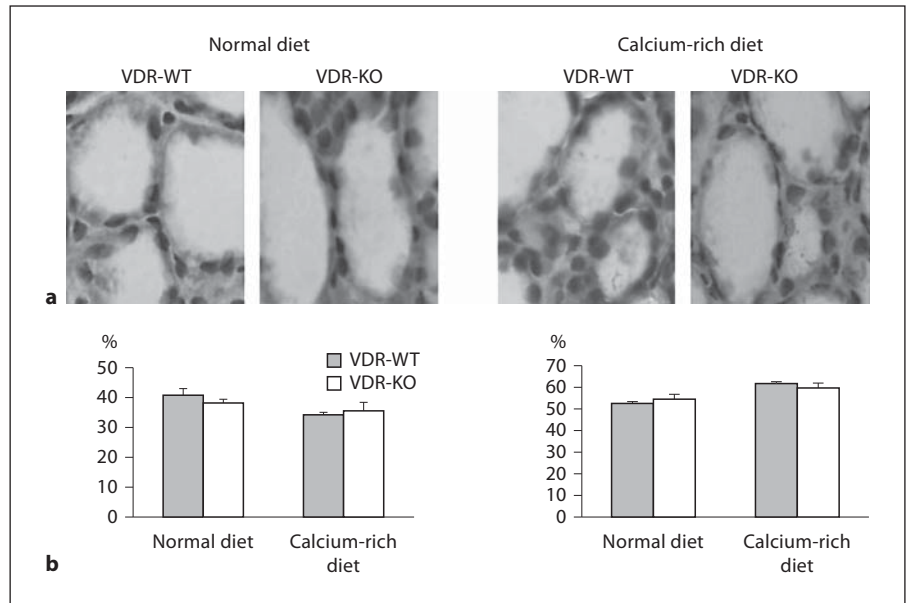


Fig. 1. The effect of VDR disruption on mouse thyroid morphology. **a** Hematoxylin and eosin staining of thyroids isolated from VDR-WT and -KO mice on normal and calcium-rich diets. Original magnification $\times 40$. **b** Relative volume of colloid (left) and epithelium (right) in VDR-WT and -KO mice on normal and calcium-rich diets according to the point-counting method.

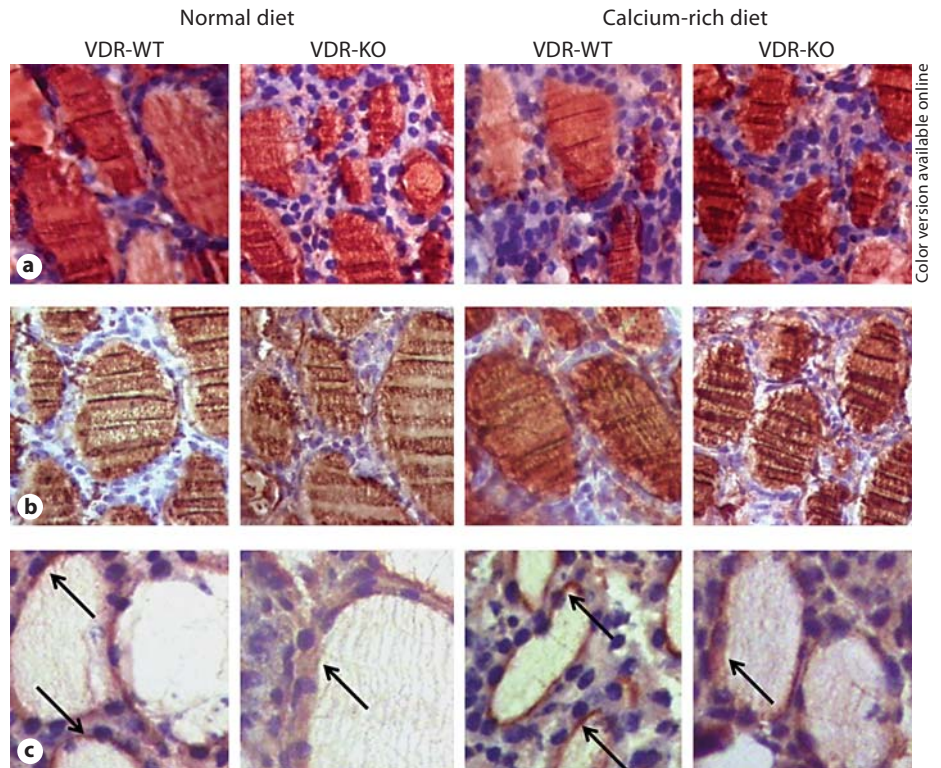


Fig. 2. Immunohistochemical staining of thyroid sections ($7 \mu\text{m}$) of VDR-WT and -KO mice on normal and calcium-rich diets. Sections were stained for Tg-I (**a**), T4 (**b**) and Duox (**c**). Immunostaining for Tg-I and T4 was similar in all groups. Immunostaining for Duox tended to be more apical in the VDR-WT mice and more in the cytoplasm in the VDR-KO mice (indicated by arrows), irrespective of the diet. Original magnification $\times 40$.

Thyocyte and C Cell Function in Mice in the Absence of VDR Signaling

Thyocyte functionality was assessed by immunostaining of thyroid sections of VDR-WT and -KO mice for Tg-I, T4 and Duox. As shown in figure 2, no difference

was observed for Tg-I and T4 between VDR-WT and -KO mice on either diet. Duox tended to be located more at the apical pole of the thyrocytes in WT mice and more in the cytoplasm in the VDR-KO mice, again irrespective of the diet. As altered expression of Duox could be caused by

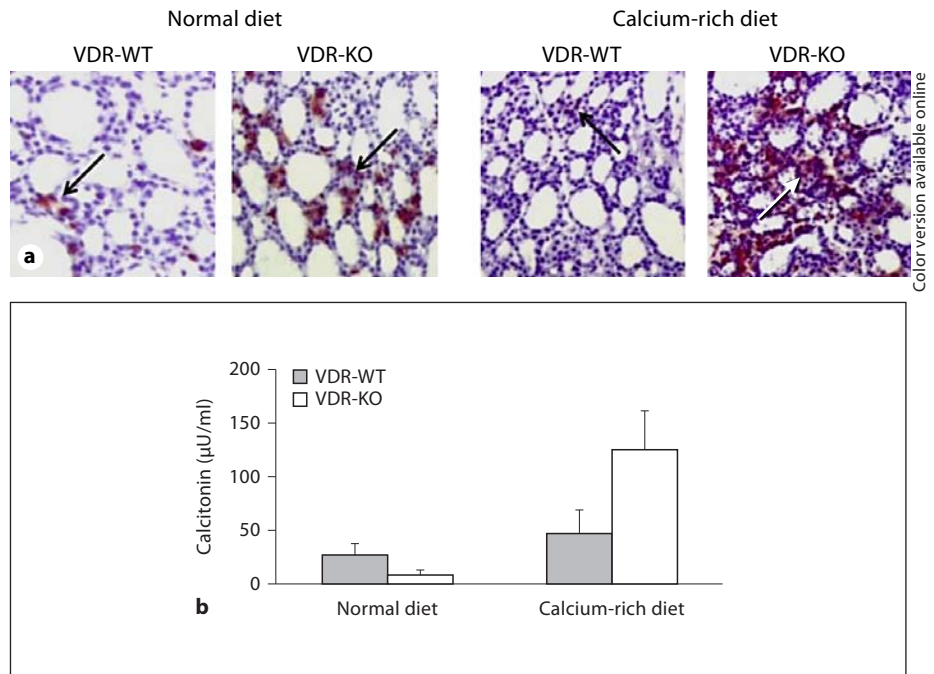


Fig. 3. Calcitonin expression in thyroid tissue and serum of VDR-WT and -KO mice on a normal and calcium-rich diet. **a** Immunohistochemical staining for calcitonin on thyroid sections (7 μm) showing a clearly higher expression (indicated by arrows) in VDR-KO mice compared to VDR-WT mice, irrespective of the diet. Original magnification ×10. **b** Serum calcitonin levels were elevated in VDR-KO mice fed a calcium-rich diet, but not in VDR-KO mice on a normal diet, suggesting that calcium constitutes a major trigger for calcitonin secretion.

oxidative stress, staining for 4-HNE (a marker of oxidative stress) was additionally performed, as well as for caspase-6 (a marker of apoptosis). Both 4-HNE and caspase-6 staining were not different between the groups (data not shown). Under the normal diet, serum TSH was not different between VDR-WT and -KO mice (0.39 ± 0.005 and 0.53 ± 0.08 μU/ml, respectively), but under the calcium-rich diet TSH was slightly lower in the VDR-KO mice (0.33 ± 0.02 μU/ml) compared to the VDR-WT mice (0.58 ± 0.1 μU/ml; $p = 0.021$).

C cell function was studied by calcitonin staining of thyroid sections, revealing a strongly increased calcitonin expression in VDR-KO compared to the WT mice, irrespective of the diet (fig. 3a). Subsequent serum calcitonin measurements confirmed increased serum calcitonin levels in VDR-KO mice (125.1 ± 36.4 ng/ml) compared to VDR-WT mice (46.4 ± 22.8 ng/ml) fed a calcium-rich diet. However, this increase was not observed in hypocalcemic VDR-KO mice fed a normal diet (fig. 3b).

In Humans, Vitamin D Status Does Not Influence Basal Serum Calcitonin Levels

Since C cell function was clearly altered in the absence of normal $1,25(\text{OH})_2\text{D}_3$ -VDR signaling in mice, we investigated whether these findings could also be relevant for humans. More specifically, we studied whether vitamin D deficiency – a widespread health problem – has an in-

fluence on basal serum calcitonin levels. We studied serum calcitonin levels in males and females with low and high serum 25-OHD_3 levels (≤ 10 and ≥ 40 ng/ml, respectively) separately. As can be observed in table 2, 25-OHD_3 serum levels were significantly different in both the male and the female patient groups. Furthermore, both the vitamin D-deficient and vitamin D-sufficient groups were normocalcemic and comparable for age, renal function and proton pump inhibitor use.

As shown in figure 4, basal serum calcitonin levels were not different between the groups with low or high 25-OHD_3 , neither when studying both sexes separately, nor when evaluating both sexes grouped. Also, after exclusion of patients with renal failure ($\text{eGFR} < 30$ ml/min/ 1.73 m²) or users of proton pump inhibitors (both known confounders increasing serum calcitonin), no difference in basal serum calcitonin level was observed between the vitamin D-deficient and vitamin D-sufficient group (data not shown).

Discussion

In the present study we investigated the role of the VDR in normal thyroid physiology by studying systemic VDR-KO mice in comparison to WT age-matched and sex-matched littermates. Thyrocyte function was not al-

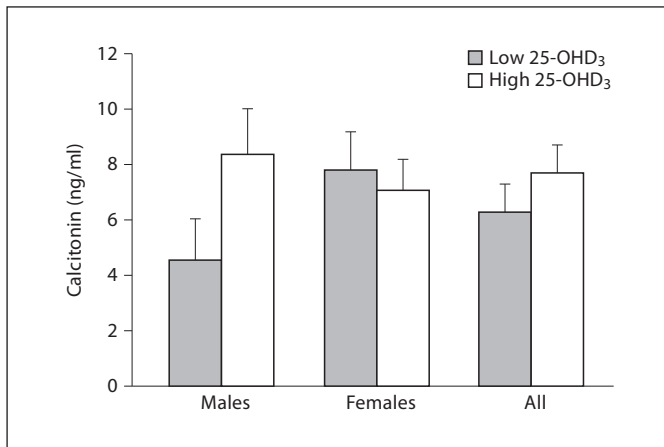


Fig. 4. Serum calcitonin levels in vitamin D-deficient (low 25-OHD₃) and vitamin D-sufficient (high 25-OHD₃) patients without history of known thyroid or parathyroid disease, medullary cancer or neuroendocrine cancer. Serum calcitonin levels were not significantly different, neither when studying both sexes separately, nor when both sexes were grouped.

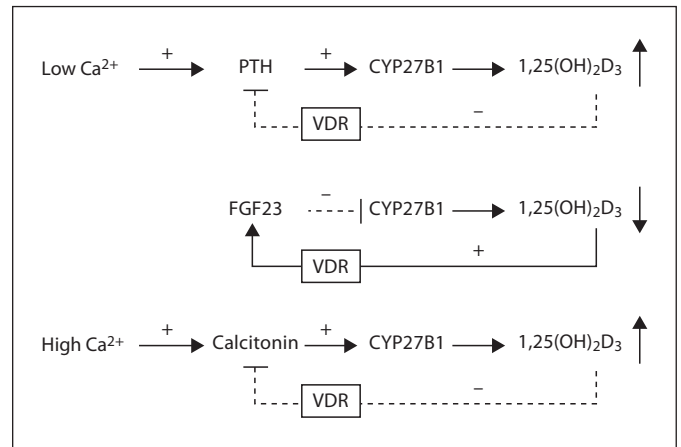


Fig. 5. Overview of 1,25(OH)₂D₃ homeostasis maintained by PTH, FGF23 and calcitonin. Low calcium levels trigger PTH release which stimulates CYP27B1. High calcium levels stimulate calcitonin release, also stimulating CYP27B1. FGF23 represents the negative regulator of CYP27B1. PTH, FGF23 and calcitonin are regulated by 1,25(OH)₂D₃ via the VDR. Stimulating effects are indicated with full arrows, inhibitory effects are indicated with dashed lines.

tered but C cell activity was clearly increased in the absence of the VDR, confirming that 1,25(OH)₂D₃ negatively regulates calcitonin via the VDR.

Supraphysiological doses of active vitamin D have been described to inhibit proliferation and reduce the severity of autoimmunity in case of underlying malignant or autoimmune thyroid disease, respectively [5, 6, 11]. In the present study, global thyroid morphology was unchanged in VDR-KO mice, also after correction of the hypocalcemic state, and no signs of autoimmune thyroiditis or thyroid neoplasia were present. Further analysis of thyrocyte functionality by studying the expression of T4 and Tg-I showed no differences. Duox was located more in the cytoplasm (instead of apically in the VDR-WT mice), possibly as a result of oxidative stress. However, no signs of increased oxidative stress (4-HNE) or apoptosis (caspase-6) were observed. Serum TSH was not different under the normal diet, but was slightly lower under the calcium-rich diet. A similar observation was made by others in rats with severe vitamin D deficiency, but serum T4 levels remained unchanged [31]. These results suggest that the 1,25(OH)₂D₃-VDR signaling pathway has a redundant role in normal thyrocyte development and function in rodents, although a mild effect on the TSH setpoint is possible.

Table 2. Characteristics of patients with low and high serum 25-OHD₃ level

	Low vit. D	High vit. D	p value
Serum 25-OHD ₃ , ng/ml			
Males	7.1 ± 0.74	49.3 ± 2.5	<0.0001
Females	6.6 ± 0.59	53.6 ± 4.8	<0.0001
Age, years			
Males	60.8 ± 4.0	59.0 ± 4.6	0.78
Females	58.7 ± 6.8	54.5 ± 3.0	0.58
creatinine, mg/dl			
Males	1.5 ± 0.5	2.3 ± 0.6	0.32
Females	1.1 ± 0.4	1.0 ± 0.1	0.78
eGFR, ml/min/1.73 m ²			
Males	49.2 ± 8.9	35.3 ± 8.0	0.28
Females	59.5 ± 12.0	50.3 ± 7.3	0.87
calcium, mg/dl			
Males	8.8 ± 0.2	9.0 ± 0.2	0.56
Females	9.1 ± 0.2	9.4 ± 0.2	0.21
PPI use, %			
Males ^a	45 (5/11)	42 (5/12)	0.85 ^b
Females	33 (4/12)	17 (2/12)	0.35 ^b

PPI = Proton pump inhibitor.

^a PPI use was not known in 1 male with low vitamin D.

^b χ^2 test.

Second, we studied the role of the VDR in parafollicular C cell physiology. The enzyme CYP27B1, responsible for the final activation of 25-OHD₃ into active 1,25(OH)₂D₃, is known to be positively regulated by PTH and calcitonin, and negatively regulated by FGF23. The transcription factor C/EBPβ and the SWI/SNF chromatin remodeling complex mediate CYP27B1 transcription induced by calcitonin [23]. In the present study we observed clearly increased thyroidal calcitonin expression in VDR-KO mice, independent from the serum calcium level. In the normocalcemic mice the increased calcitonin expression at the C cell level was paralleled by increased serum calcitonin levels. This was not observed in VDR-KO mice fed a normal diet, which is most probably explained by their hypocalcemic status inhibiting secretion of calcitonin by the C cells. In a rat model, it has been established by others that calcium constitutes the main regulator for calcitonin secretion, but that 1,25(OH)₂D₃ and not calcium constitutes the main regulator for calcitonin transcription [25, 32]. We hereby demonstrate for the first time that the inhibitory action of 1,25(OH)₂D₃ on calcitonin production acts via the VDR. Due to the absence of VDR signaling, the inhibitory effect of 1,25(OH)₂D₃ on C cells and thus the physiological negative feedback control is lost. Thyroiditis and renal insufficiency were ruled out as potential other causes for increased calcitonin by increased bystander C cell activation and decreased renal clearance, respectively. Furthermore, Egrise et al. [31] described that rats fed a vitamin D-deficient diet showed strongly increased thyroidal calcitonin mRNA expression, which suggests that deficiency of the ligand for VDR also results in uncontrolled calcitonin expression in rodents. Whether chronically defective signaling could eventually lead to C cell proliferation is not known. Thus, as summarized in figure 5, we demonstrate in the present study that besides the known positive regulation of CYP27B1 by PTH and its negative feedback via 1,25(OH)₂D₃-VDR signaling, calcitonin (the other known positive regulator of CYP27B1, but mainly active under normocalcemic to hypercalcemic conditions) is also negatively controlled by 1,25(OH)₂D₃ via the VDR. FGF23 represents the negative regulator of CYP27B1, with a positive feedback by 1,25(OH)₂D₃ via the VDR.

In the last part of the study, we investigated whether increased calcitonin caused by defective 1,25(OH)₂D₃-VDR signaling as observed in rodents also applies to humans. If so, this would imply that the vitamin D status and also VDR polymorphism might influence the basal calcitonin level. As vitamin D deficiency is very prevalent, this could have important clinical consequences.

On the other hand, it is known that C cell physiology, in contrast to thyrocyte physiology, is largely different in rodents as compared to humans [33, 34]. We found no increased basal serum calcitonin levels in a cohort of patients with severe vitamin D deficiency compared to patients with vitamin D sufficiency (all patients were normocalcemic and matched for sex, age, renal function, use of proton pump inhibitors). The variable comorbidity of the patients, however, represents a limitation of this study.

In conclusion, VDR disruption did not influence thyroid morphology or thyrocyte function in mice. However, VDR constitutes an important determinant in C cell physiology in mice, negatively controlling calcitonin. In a pilot study in patients, there was no evidence for alterations of serum calcitonin levels in case of very low or high levels of serum 25-OHD₃. These findings need to be confirmed in a study in healthy individuals, preferably by a prospective randomized placebo-controlled clinical trial, evaluating serum calcitonin before and after vitamin D supplementation along with serum levels of 25-OHD₃, 1,25(OH)₂D₃, PTH, FGF23 and VDR polymorphism. At present, there is no indication for a systematic evaluation of the patient's vitamin D status along with a serum calcitonin measurement.

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Disclosure Statement

The authors declare that no competing financial interest exists.

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