Modulation of airway epithelial cell function by vitamin D in COPD

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CHAPTER

General Introduction and Outline of the Thesis

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Introduction

Vitamin D is a well-recognized for its systemic role in calcium absorption and bone mineralization. In the airways, vitamin D enhances airway epithelial cell homeostasis and is a modulator of both innate and adaptive immune responses (1). The airway epithelium is the front line of the lung's host defense and its main function is to clear and protect the airways from hazardous inhaled substances such pollutants and pathogens (2). These protective airway epithelial cell functions could be affected by for example genetic alterations and environmental insults in early life and/or the long-term exposure to inhaled toxicants, such as caused by cigarette smoking. Consequently, this may increase the susceptibility towards infections and eventually lead to (chronic) inflammation and aberrant immune responses, epithelial remodeling and repair (3, 4). Furthermore, studies have now shown that the airway epithelium from patients with chronic inflammatory lung diseases may differ from healthy subjects, and show signs of altered epithelial differentiation and aberrant repair (4-6). These findings support the hypothesis that the airway epithelium plays a central role in the pathogenesis of chronic inflammatory lung diseases such as asthma and chronic obstructive pulmonary disease (COPD).

Vitamin D deficiency was classically known to be solely associated with bone diseases such as rickets until it gradually became evident that vitamin D deficiency was also associated with many other diseases including chronic inflammatory lung diseases such as asthma and COPD (1, 7-9). Several *in vitro* studies have already indicated that the active form of vitamin D [1,25 dihydroxy-vitamin D ($1,25(OH)_2D$)] might protect the airway epithelium against injury by promoting integrity of the epithelial barrier, dampening immune responses and via the induction of the antimicrobial peptide (AMP) hCAP18/LL-37 (1). However, the precise impact of $1,25(OH)_2D$) on the airway epithelium in a chronic inflammatory environment as observed in chronic lung diseases remains to be defined.

The Airway Epithelium

The lungs are in close contact with the external environment due to the large volumes of air inhaled on a daily basis. The airway epithelium lines the conducting airways and removes and eliminates potentially harmful substances and pathogens to protect the alveoli, where gas exchange occurs, from injury. The epithelium that lines the conducting airways is composed of a pseudostratified cell layer that consists of several epithelial cell types of which the major cell-types are ciliated

cells, secretory and basal cells (10). The basal cells are regarded as the main progenitor cells, that are able to renew and differentiate into intermediate cells followed by end-stage differentiation into secretory- or ciliated cells (5, 10). The ciliated cell is the main cell type of the airway epithelium that transports mucus produced by the submucosal glands and the goblet cells of the surface epithelium out of the airways through highly coordinated ciliary beating, generating a wavelike movement across the epithelial surface (11). Activation of the transcription factor forkhead box J1 (FOXJ1) is involved in the formation of ciliated cells (11), whereas Notch-signaling and activation of SAM pointed domain–containing ETS transcription factor (SPDEF) are the main inducers for secretory cell differentiation (12, 13). The goblet- and club cells are the secretory cells of the conducting airways. Whereas goblet cells are more prominent in the trachea and bronchi, club cells are most the predominant secretory cell type in the terminal and respiratory bronchioles (small airways). Goblet cells predominantly secrete gel-forming mucins such as mucin-5AC (MUC5AC) that trap particles in the gel-matrix to be removed through the mucociliary escalator, which is dependent on proper mucin-hydration and function of ciliated cells (14). Club cells are present throughout the airways and are the main source of club cell protein (CC16), which has been shown to contribute to epithelial homeostasis and to reduce inflammation and might therefore have protective features against development and progression of COPD (15). Furthermore, club cells also secrete antimicrobial compounds, surfactant proteins and mucins such as mucin-5B (MUC5B), which are known regulators of respiratory host defense and they express cytochrome P450 (CYP) enzymes such as CYP2F2 that neutralize xenobiotics (5, 15-17). The fact that CYP2F2 metabolizes some toxins into even more harmful substances might explain why club cells are more sensitive to these toxins than other airway epithelial cell types (Figure 1) (18).

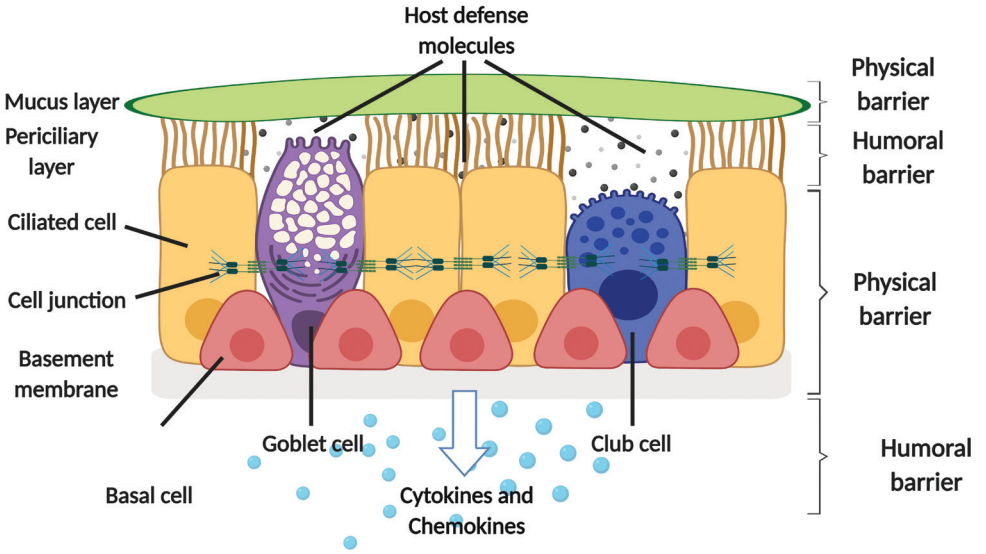

Figure 1. The airway epithelium: cells that line and constitute the physical- and humoral barrier of the conducting airways. Physical defenses are provided by the mucociliary clearance and cell junctions forming the physical epithelial barrier, whereas humoral defenses are provided by the secretion of host defense molecules such as interferons (type I and III), antimicrobial peptides and proteins, cytokines, chemokines and lipid mediators. These mediators are also involved in regulating both innate and adaptive immune responses by attracting and activating immune cells in the submucosa.

Airway epithelial innate host defense involves a physical barrier and a humoral barrier (Figure 1). The physical barrier of the epithelium is maintained by its tight- and adherens junctions and mucociliary clearance of trapped particles and pathogens (3). In chronic inflammatory airway diseases such as asthma and COPD, both of these defenses are compromised, which contributes to increased susceptibility towards infections (19). Furthermore, the ability to repair damage upon infection or exposure to toxicants is also impaired, and it has been hypothesized that aberrant repair results in remodeling of the airway epithelium such as reduced numbers of ciliated cells, goblet cell hyperplasia or epithelial-to mesenchymal transition (EMT) (20). EMT is a process characterized by a transformation of differentiated epithelial cells into mesenchymal cells (21). Furthermore, airway host defense is maintained via the humoral barrier provided by the secretion of host defense molecules such as antimicrobial proteins and peptides (AMPs), secretory immunoglobulin A (sIgA) and reactive oxygen species (ROS) (3). Furthermore upon activation of pattern recognition receptors (PRRs),

interferons, cytokines, chemokines and lipid mediators are released to promote viral clearance and to recruit and activate innate and adaptive immune cells (22, 23). AMPs have, in addition to their broad-spectrum antimicrobial activity, also the ability to modulate immune responses and to promote wound repair (24). AMPs are either constitutively expressed at high levels or their expression can be induced upon activation of PRRs, cytokine and growth factor receptors, and by other mediators such as $1,25(OH)_2D$ (25). In addition to AMPs, ROS also have antimicrobial features and are generated in airway epithelial cells by dual oxidases (DUOX), which are localized on the apical plasma membranes (26, 27). Epithelial clearance of viruses is generally promoted via the expression and release of type I and III interferons, which induces expression of a range of proteins that selectively interfere with virus replication, protein synthesis, or protein trafficking (23). In the chronic inflammatory lung diseases, aberrant secretion of AMPs and impaired expression of interferons contributes to defective clearance of pathogens, despite the increased production of ROS and influx of inflammatory cells (3, 23, 25, 28).

Chronic Obstructive Pulmonary Disease (COPD) and Exacerbations

COPD is a progressive lung disease characterized by chronic obstruction of airflow that interferes with normal breathing and is not fully reversible. The primary cause of COPD is exposure to noxious particles or gases such as tobacco smoking and environmental exposures to biomass fuels or air pollution, but also host factors such as genetics, abnormal lung development and aging may contribute to COPD development (29). COPD is a major cause of chronic morbidity and is currently the 3rd leading cause of death in the world (29, 30). Exacerbations of COPD are episodes of acute worsening of symptoms that require additional therapy, accelerate disease progression and are a substantial burden on health-care systems worldwide through their effects on morbidity and mortality (29, 31). COPD exacerbations are mainly associated with by respiratory viral- and bacterial infections or by air pollution (31). As discussed in the previous paragraph, dysfunctional epithelial innate defense in the lungs of COPD patients likely contributes to the increased susceptibility towards bacterial and viral infections, and thereby to COPD exacerbations. Moreover, multiple studies have now demonstrated that COPD patients generally have lower serum 25(OH)D-levels, which are associated with an increased number and more severe exacerbations (8, 32). Studying the role of vitamin D in COPD exacerbations and airway epithelial innate immune defenses might therefore provide additional insight into the potential benefit of monitoring

vitamin D status of these patients and supplementation of patients with a vitamin D deficiency.

Vitamin D Deficiency

Vitamin D is a pleiotropic hormone that is well known for its role in regulating calcium (Ca^{2+}) and phosphate (PO_4^{2-}) homeostasis and bone mineralization. The receptor for 1,25$(OH)_2$D (VDR) is however expressed in more than 40 tissues and regulates a large number of genes (approximately 0.8–5% of the total genome) (33). As a result, 1,25$(OH)_2$D affects several cellular processes including proliferation, DNA repair, differentiation, apoptosis, membrane transport, metabolism, cell adhesion, and oxidative stress (33, 34). Vitamin D deficiency [serum 25(OH)D < 50 nmol/L (35)] affects more than 30% of the children and adults worldwide and is linked to bone diseases such as rickets and osteoporosis, but also associated with many other diseases including cancer, type 2 diabetes, cardiovascular diseases, Alzheimer's' disease, muscle myopathy, multiple sclerosis, inflammatory bowel disease (IBD), psoriasis, and chronic inflammatory lung diseases including asthma and chronic obstructive pulmonary disease (COPD) (1, 7-9).

Metabolism of Vitamin D

Vitamin D enters the body either via food intake or as a result of its synthesis in the skin under the influence of UV-light (Figure 2). In the intestine, vitamin D_2 or vitamin D_3 enters the circulation actively from the lumen by apical membrane transporters or by passive diffusion through enterocytes (36). In the skin, UVB radiation triggers conversion of the cutaneous reservoir of 7-dehydrocholesterol into pre-vitamin D_3. After isomerization of pre-vitamin D_3 into vitamin D_3, this secosteroid is removed from the skin through binding to the vitamin D binding protein (VDBP) and transported into the circulation (37). This VDBP-bound vitamin D_2 or vitamin D_3, which has a half-life of one day, is transported to the liver where it is converted by vitamin D-25-hydroxylases (CYP2RI and CYP27A1) into 25-hydroxy-vitamin D [25(OH)D]. However, recent studies showed that also other cell types such as airway epithelial cells, keratinocytes, monocytes/macrophages and intestinal epithelial cells express CYP2RI and CYP27A1, and thus are able to (locally) convert vitamin D_3 into 25$(OH)D_3$ (38, 39). 25(OH)D (both 25$(OH)D_2$ and 25$(OH)D_3$) is the main circulating form of vitamin D, which has a half-life of 20 days and its levels are used to assess vitamin D status in the clinic (40). This inactive 25(OH)D needs to be

converted into the active 1,25 dihydroxy-vitamin D ($1,25(OH)_2D$) by 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) in the kidney, locally in tissues or in several types of immune cells (41-44). $1,25(OH)_2D$ binds the nuclear vitamin D receptor (VDR), which heterodimerizes with the retinoic acid receptor (RXR) to interact with vitamin D response elements (VDREs) that are present on the promoter region of vitamin D target genes (33, 45). The VDR-RXR-complex needs additional corepressors (NCoR, SMRT) and/or co-stimulators (SRC-1, CBP, MED1) to silence or initiate gene transcription (46, 47). In addition to promoting or suppressing gene expression via binding of $1,25(OH)_2D$ to the nuclear (nVDR) and RXR, 1,25(OH)2D furthermore interacts with membrane-bound VDR (mVDR), known as $1,25(OH)_2D$-membrane-associated, rapid response steroid-binding protein (1,25D-MARRS). mVDR is present in the caveolae and induces rapid non-genomic responses through e.g. the generation of second messengers such as Ca^{2+} and/or activation of proteins kinases such as mitogen-activated protein kinases (MAPK) (48). Furthermore, $1,25(OH)_2D$ regulates its own negative feedback by several mechanisms, for example via direct induction of the catabolic enzymes 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) and CYP3A4 (49, 50). CYP24A1 is expressed in most tissues and converts both 25(OH)D and $1,25(OH)_2D$ into $23,25(OH)_2D$ or $24,25(OH)_2D$ and $1,24,25(OH)_3D$ respectively, which are further converted into inactive metabolites and excreted in the bile (49, 51). CYP3A4 is mainly expressed in the liver and small intestines and contributes to the metabolic clearance of 25(OH)D and $1,25(OH)_2D$ by converting 25(OH)D into $4\beta,25(OH)_2D$, and $1,25(OH)_2D$ into $1,23R,25(OH)_2D$ or $1,24S,25(OH)_2D$ (50). Expression of both CYP27B1 and CYP24A1 in the kidneys is tightly regulated to maintain optimal Ca^{2+} and PO_4^{2-}-levels in the circulation. When Ca^{2+}-levels are low, parathyroid hormone (PTH) is secreted by the pituitary glands, which in turn reduces Ca^{2+} excretion and reabsorption of PO_4^{2-} (52). PTH further induces expression of CYP27B1 and represses expression of CYP24A1 in the kidneys (52). This will increase the levels of $1,25(OH)_2D$ in the circulation, which promotes intestinal Ca^{2+} and PO_4^{2-} absorption (52). These elevated circulating Ca^{2+} and PO_4^{2-} levels will subsequently induce expression of fibroblast growth factor 23 (FGF-23) in osteocytes and osteoblasts and impair secretion of parathyroid hormone (PTH) by the parathyroid glands (34). In the kidneys, FGF-23 suppresses expression of CYP27B1 and induces expression of CYP24A1, thereby inhibiting the synthesis and promoting degradation of $1,25(OH)_2D$ (34) (Figure 2).

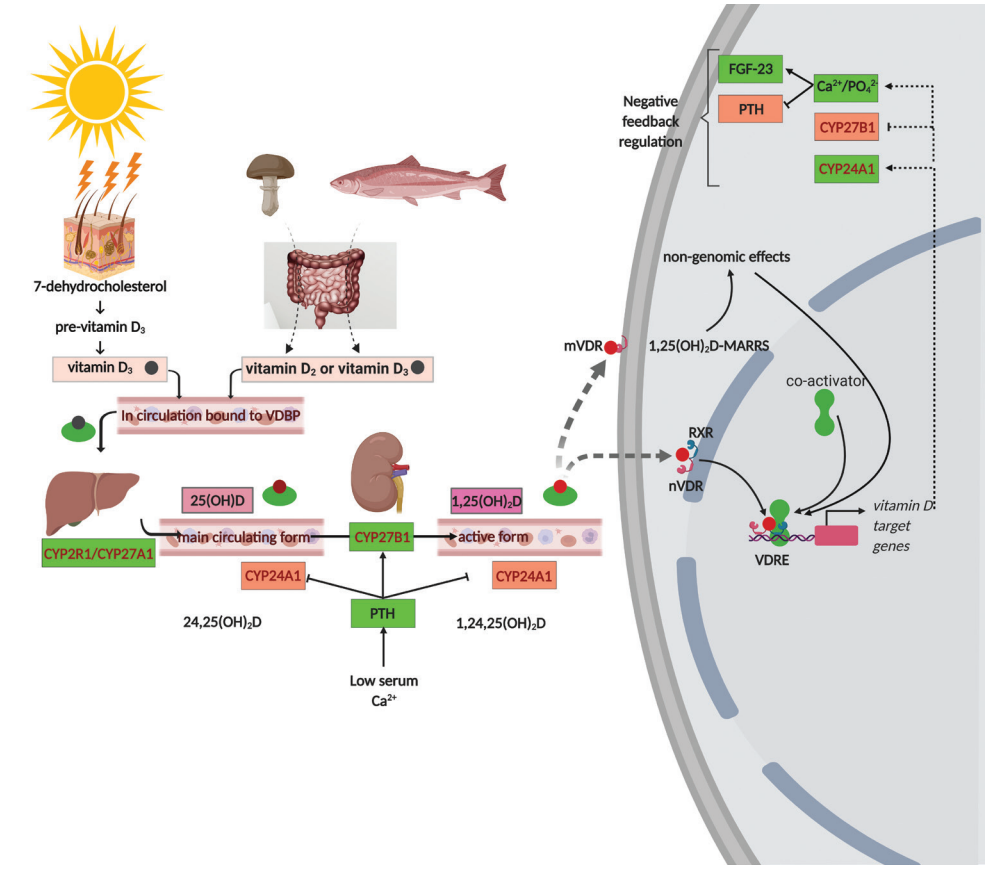

Figure 2. Endocrine vitamin D metabolism. See text for details and references.

Vitamin D and Chronic Inflammatory Lung Diseases

Systemic levels of biologically active $1,25(OH)_2D$ are tightly regulated to achieve sufficient Ca^{2+} and PO_4^{2-} levels levels for optimal bone mineralization, whereas in mucosal tissues local $1,25(OH)_2D$ activation or inactivation can result in $1,25(OH)_2D$ levels that are elevated or decreased (7). The inflamed mucosal tissues of the airways in COPD and asthma patients are constantly exposed to pathogens and to several inflammatory mediators. However, the effects of this exposure on local levels of $1,25(OH)_2D$ in mucosal tissues of the lung and gut are currently unclear. This may however be relevant since many diseases with chronic inflammation of the lung such as asthma and COPD are also associated with vitamin D deficiency (8, 53, 54). These patients have furthermore dysregulated immune responses, altered microbiome composition, impaired epithelial barrier function and aberrant

secretion of host defense molecules thereby increasing their susceptibility towards infections (55-57). Since 1,25$(OH)_2$D is involved in many of these processes, it might provide protection against these features. This may occur via various mechanisms induced by 1,25$(OH)_2$D, including the maintenance of the integrity of the mucosal barrier and the promotion of killing of pathogens (e.g. via the induction of the antimicrobial peptide hCAP18/LL-37) (1). Mechanistic studies have furthermore shown that 1,25$(OH)_2$D is an important mediator of both innate and adaptive immune responses, suggesting the importance of 1,25$(OH)_2$D in various immune-related diseases (58). COPD patients have an increased risk for vitamin D deficiency, which is associated with an increased number and more severe exacerbations (8, 32). Studying the role of 1,25$(OH)_2$D in COPD exacerbations and airway epithelial innate immune defenses might therefore provide additional insight into the potential benefit of monitoring 25(OH)D levels in these patients and supplementation of patients with a vitamin D deficiency.

Outline of the Thesis

The overall aim of the studies described in this thesis is to elucidate the role of inflammation on the protective effects of vitamin D on respiratory host defense in chronic airway diseases with a specific focus on COPD. First, an introduction into the central role of the respiratory epithelium during homeostasis and in the pathogenesis of chronic inflammatory lung diseases such as COPD is provided, followed by a general introduction into vitamin D metabolism and vitamin D deficiency in COPD patients (**Chapter 1**). In the experimental studies that are described in this thesis, the effects of vitamin D (inactive 25(OH)D and active $1,25(OH)_2D$) and inflammation are studied using *in vitro* models of primary bronchial epithelial cells. In these models, we will study the effects of toxic, inflammatory and microbial exposures such as cigarette smoke (**chapter 2**), cytokines (**chapter 3** and **4**), bacteria (**chapter 4**) and viruses (**chapter 5**) on airway epithelial host defense mechanisms with a focus on $1,25(OH)_2D$-mediated epithelial host defense mechanisms, i.e. expression of the AMP hCAP18/LL-37 (Figure 3).

Both smokers and COPD patients are more susceptible for respiratory infections and various studies have shown that cigarette smoke exposure may alter airway epithelial cell composition. We therefore first aimed to investigate in **Chapter 2** if chronic cigarette smoke-exposure during epithelial differentiation alters expression of constitutively expressed host defense molecules and host defense mechanisms through its effect on airway epithelial cell differentiation. Cigarette smoke exposure also induces expression of TGF-β1 in airway epithelial cells. This pleiotropic cytokine is elevated in COPD patients, and in addition to its ability to promote fibrosis, TGF-β1 also impairs airway epithelial host defense mechanisms. Therefore, we next focused in **Chapter 3** on investigating the effects of TGF-β1 on expression of constitutively expressed host defense molecules and on the expression of the $1,25(OH)_2D$-induced hCAP18/LL-37. We furthermore determined mechanisms underlying behind the impaired effects of TGF-β1 on respiratory host defense by investigating direct effects of TGF-β1 on *CAMP* (hCAP18/LL-37) transcription, on vitamin D metabolism and on VDR expression. In **Chapter 4**, we continued to investigate the effects of proinflammatory cytokines (TNF-α, IL-1β, IL-17A), elevated in the airways of COPD and/or in steroid resistant asthma patients, on vitamin D-metabolism and on 25(OH)D and $1,25(OH)_2D$-mediated expression of hCAP18/LL-37. To determine if the 25(OH)D and $1,25(OH)_2D$-mediated respiratory host defense was affected by exposure to proinflammatory cytokines, we assessed epithelial antimicrobial activity against nontypeable *Haemophilus influenzae*.

Chapter 5 describes the immunomodulatory effects 25(OH)D and 1,25(OH)$_2$D on virus-induced (Poly[I:C])-inflammatory responses in airway epithelial cells. In addition, the effects of Th2 inflammation -present in the airways of both allergic asthma and a subset of COPD patients- on vitamin D metabolism and 25(OH)D and 1,25(OH)$_2$D-mediated expression of hCAP18/LL-37 was investigated. To translate our findings to a more clinical level in **Chapter 6,** a study design is provided that describes a multicenter randomized controlled trial that aims to investigate if vitamin D supplementation can indeed protect against COPD exacerbations in a population of vitamin D deficient COPD patients. Finally, in **Chapter 7** a review of all the current knowledge of the effects of disease-associated factors such as inflammation and cigarette smoke exposure on availability and signaling of 1,25(OH)$_2$D in the lungs of patients with COPD and other chronic lung diseases is provided, followed by an general overview and discussion of the results that are presented in this thesis in **Chapter 8**.

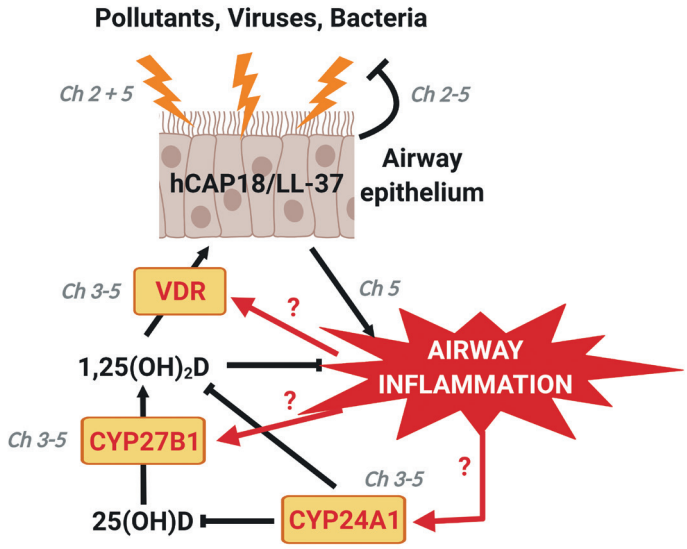

Figure 3. Unknown effects of inflammation on vitamin D metabolism and epithelial host defense in the airways. The separate components of this figure are discussed in the various chapters of this thesis, as indicated by the chapter (Ch) numbers. Vitamin D receptor, VDR; Biologically active vitamin D, 1,25(OH)$_2$D; 25-hydroxyvitamin D-1α-hydroxylase, CYP27B1; circulating inactive vitamin D, 25(OH)D; 25-hydroxyvitamin D-24-hydroxylase, CYP24A1.

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