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Vitamin D in health and disease

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Vitamin D yesterday, today, tomorrow

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In the last decade, vitamin D has become one of the most frequently ordered tests worldwide. In France, frequency of vitamin D testing has increased 10-fold in the last ten years, amounting to 150 million EUR spent for testing in private labs. In USA vitamin D has become 5th most commonly ordered test in Medicare healthcare system. Expansion of vitamin D testing is a result of many promising observational studies that have associated vitamin D concentration with health benefits in cardiovascular diseases, cancer, diabetes, fertility and many others. Number of published papers increases daily, however, all are not confirming initial findings.

Based on the Croatian guideline for prevention, detection and therapy of vitamin D deficiency in adults, issued in 2016 by several professional societies, concentrations lower than 30 nmol/L are considered as extreme deficiency, < 50 nmol/L as deficiency and < 75 nmol/L as insufficiency. Data collected from the Department of Clinical Chemistry, Sestre milosrdnice University Hospital Center showed that out of 11,863 patients tested for vitamin D in 2018, only 2901 (26%) had vitamin D concentration > 75 nmol/L. Recommended daily intake of vitamin D for adults is 600 IU, and if sufficient amount is not ingested by food, supplements should be taken.

Usage of dietary supplements has become more increasing in recent years. In USA, in 2018 market share for vitamins has accounted for 37.64 billion USD. Large European multicentre study published in 2018 on patient's knowledge and awareness about the effect of the over-the-counter drugs and dietary supplements on laboratory test results, has revealed some alarming issues. More than two thirds of patients are taking at least one dietary supplement, but they are usually not reporting the usage neither to their physicians nor to the laboratory staff. Our recent study has shown

that methods for vitamin D measurement might react differently when vitamin D is measured in patients who are taking vitamin D supplements and those who are not. Usage of any supplements that might affect laboratory testing should be reported to laboratory staff.

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Mechanism of action and physiologic function of vitamin D

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Vitamin D and its metabolites are steroid hormones and hormone precursors. About 80% of vitamin D₃ is produced by UVB irradiation of the 7-dehydrocholesterol in the skin, and the remainder comes from the diet and food supplements mainly in the form of vitamin D₂ (with the exception of fatty fish). Vitamin D₂ is produced by UVB irradiation in plants and fungi. Regardless of origin, vitamins D₂ and D₃ are biologically inactive before two hydroxylation processes in the liver and the kidney preceding the formation of the biologically active form of vitamin D, 1,25(OH)₂D.

All genomic actions of 1, 25(OH)₂D are mediated by vitamin D binding protein (VDBP). Vitamin D and its metabolites are transported in the circulation by VDBP, and when reaching their target cells, they dissociate from the VDBP, enter the cells and interact with a nuclear vitamin D receptor (VDRn). VDRn was detected in various tissues and cells and functions as a transcription factor. Liganded VDRn forms a heterodimeric complex with Retinoid-X-Receptor (RXR) and up regulates or down regulates the expression of target genes through binding to the responsible elements in the promoter region of the regulated gene (VDREs) initiating a formation of an assembly of nuclear transcription factors.

However, some effects of vitamin D in target cells are too rapid to be explained by stimulation of gene expression. It is recognized that 1,25(OH)₂D also act through non-genomic actions that are mainly manifest as the activation of intracellular signalling molecules and consequently transcription factors that bind to the VDRE. Another non-genomic action of 1,25(OH)₂D includes the regulation of VDR binding to target receptors such as STAT1 and IKK β . Non-genomic effects of 1,25(OH)₂D are mediated by membrane VDR (VDRm).

Biological effects of vitamin D are well known. The major targets of 1,25(OH)₂D are intestine, kidney and bones, where, together with other calciotropic hormones, maintains calcium balance. In addition to classic actions related to mineral homeostasis, vitamin D has novel actions in cell proliferation and differentiation, regulation of the innate and adaptive immune system, preventive effects on neurodegenerative and cardiovascular diseases, and antiaging effects.

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Effect of preanalytical factors on vitamin D concentration

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In the last few years requests for vitamin D determination is increasing. The main reason for such rise is that vitamin D deficiency proved to be associated with various diseases, especially bone metabolic diseases, but also cardiovascular, autoimmune, neurodegenerative diseases, diabetes and some cancers. In order to be sure that deficiency is

discovered, vitamin D concentration should be determined accurately and precisely. For accurate definition of vitamin D deficiency, it is necessary to know all, or as much as possible, preanalytical issues that could affect results or interpretation of results.

Published studies proved that vitamin D metabolite 25-hydroxy vitamin D 25(OH)D is very stable analyte in almost all storage conditions. Concentration of 25(OH)D is stable for days at room temperature, for years at - 20°C, and even multiple freeze-thaw cycles do not change results. Both serum and plasma can be used for measurement of 25(OH)D concentration, without statistically significant difference in results.

As declared by the commonly used manufacturers, only high levels of endogenous interferences can affect results, for example, haemolysis above 2 g/L of free haemoglobin, ictericia above 342 μ mol/L or high lipemia (with triglycerides above 3 mmol/L).

However, due to characteristic pathway and metabolism of vitamin D, some specific preanalytical issues are known. Concentration of vitamin D could be affected with amount of UV light, depending on seasonal or geographical variations, amount of exposure to sun, or sunscreen protection. Moreover, skin type and colour could also produce different concentration of active form of vitamin D in the blood. Women usually have lower concentration of vitamin D than men. Furthermore, different dietary habits, as well as body mass index influence on vitamin D concentration. A great number of patients take some food supplements with vitamin D, which could also affect measurement accuracy.

It is very important to be aware of all of these, and some others, preanalytical factors that could affect interpretation of results and lead to possible misdiagnosis of vitamin D deficiency.

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Analytical challenges in determining vitamin D

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General acceptance of serum total 25-hydroxyvitamin D (25(OH)D) as the best biomarker of an individual's vitamin D status has resulted in the development of several specific and sensitive commercial assays over the past 20 years. Its hydrophobic nature, small circulating concentrations, ability to bind to lipids and vitamin D binding proteins (VDBP), and presence of multiple vitamin D metabolites in bloodstream have defined 25(OH)D as a "difficult analyte". Precise and accurate measurement of 25(OH)D concentrations is challenging, and large variations exist between different assay methodologies. Such variations depend on several factors: different methods of vitamin D extraction and deproteinization, antibody cross-reactivity with epimers and/or other vitamin D metabolites, and presence of isobaric compounds or matrix interferences. Assays based on liquid chromatographic techniques (LC) or mass spectrometry (MS) are more accurate than the automated chemiluminescent immunoassay-based methods. Since the above mentioned methods are usually based on achiral chromatographic techniques, they cannot distinguish between 25(OH)D₃ and its 3-epimer, or other isobaric compounds (e.g. 7- α -hydroxy-4-cholesten-3-one, an endogenous precursor of bile acids) resulting in a slight overestimation of concentrations. Currently isotope-dilution LC/MS-MS is considered the gold standard method. The large discrepancies between LC-MS methods and immunoassays, but also among different immunoassay methods, are mainly due to differences in cross-reactivity with various vitamin D metabolites, which accounts for a significant proportion of total 25(OH)D. Although immunoassays do not detect 3-epi-25(OH)D₃, generating specific antibodies against small antigenic molecules such as 25(OH)D is difficult, and cross-reactivity with 24,25(OH)₂D₃ and other vitamin D me-

tabolites is common. While some immunoassays cannot detect 25(OH)D₂, those that can are unable to distinguish between 25(OH)D₂ and 25(OH)D₃. Strong binding between the lipophilic 25(OH)D and VDBP creates competition with the capturing antibody in the assays where 25(OH)D and VDBP are not completely separated. Furthermore, the discordance between 25(OH)D values from different assays is magnified by differences in standardization of each assay. As the number of 25(OH)D methods increases, it will be essential to carefully standardize and harmonize all available methods to enable the development and use of evidence-based guidelines. Fortunately, the Vitamin D Standardization Program (VDSP) is making substantial progress towards both goals.

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Vitamin D in extravascular body fluids

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Vitamin D is included in many biological processes, especially calcium and phosphate homeostasis, but also in modulation of immune system. Diagnostic potential of serum vitamin D has become subject of many researches, and measurement of its serum concentrations has been included in clinical practice. However, due to its role in physiological processes, significance of vitamin D in some extravascular fluids has been investigated. To apply appropriate method in extravascular fluid of interest, it is important to understand metabolism of vitamin D. Inactive form undergoes first hydroxylation process in the liver and it is converted to 25(OH)D - major circulating form after which is converted into active 1,25(OH)₂D in kidneys. Most of 25(OH)D form is bound to vitamin D binding protein (VDBP) which is reabsorbed by tubular reuptake mediated by receptors. Excess of vitamin D undergoes conjugation processes and its conju-

gates are excreted by urine. Any tubular damage will decrease plasma vitamin D due to impaired tubular reabsorption. Measuring of VDBP in urine could give insight into vitamin D loss by kidneys and determination of vitamin D glucuronides might improve understanding of vitamin D metabolism. Vitamin D expresses its function through vitamin D receptors which are abundantly expressed on brain tissue. Consequently, presence of those receptors and modulator role on immune system, raised interest in measuring vitamin D in cerebrospinal fluid in patients with Alzheimer disease, and multiple sclerosis. Modulator effect on cells of innate and adaptive immune cells led to hypothesis that vitamin D could be increased in pleural effusion due to infection. Similar was assessed with peritoneal fluid where serum-ascites vitamin D gradient was tested in order to diagnose spontaneous bacterial peritonitis. Synovial macrophage and fibroblasts are included in 25(OH)D metabolism, which increased interest for vitamin D determination in synovial fluid in patients with rheumatoid arthritis. Concentrations of vitamin D have also been investigated in mother's milk to examine nutritional value for new-borns and saliva is interesting due to its availability as a sample. Although limited, research of vitamin D concentrations in extravascular fluid have potential as diagnostic tool in many conditions.

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Vitamin D and infertility

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The importance of vitamin D and its isoforms; ergocalciferol and cholecalciferol, is well known for its role in calcium homeostasis and bone mineralization. Vitamin D receptors (VDR) and enzymes required for the production of active form are expressed in numerous human cells and tissues,

which links vitamin D insufficiency or deficiency to many diseases such as cancer, autoimmune, infectious and cardiovascular diseases and diabetes mellitus. Among all, VDR is expressed in ovarian tissue, uterus and placenta as well.

New theories of vitamin D effect on reproductive health have been proposed, in order to improve infertility treatment.

Although results are still inconclusive, existing data suggest the possible beneficial role of vitamin D in fertility. Besides that, many studies imply that vitamin D may as well have important role in pregnancy outcomes. Adverse pregnancy outcomes including preterm birth, gestational diabetes and preeclampsia have been observed in women with vitamin D deficiency. Also, vitamin D deficiency is thought to have several negative effects on human fertility. Evidence suggests a correlation between vitamin D and ovarian reserve markers, in particular, with anti-Mullerian hormone (AMH), suggesting that its deficiency may be associated with lower ovarian reserve in late reproductive aged women.

Besides, vitamin D might have beneficial effect on metabolic and hormonal parameters of PCOS and endometriosis, which are one of the most common reasons of female infertility. Although the exact mechanism how vitamin D affects PCO and endometriosis is not yet known, several explanations have been proposed.

The majority of studies demonstrated a positive relationship between higher vitamin D status and IVF outcome. Recent data imply that vitamin D deficiency has impact on pregnancy success in women undergoing day 5 single embryo transfer (SET). The lower clinical pregnancy rates were attributed to a negative effect of vitamin D deficiency on endometrial receptivity.

Interestingly, epidemiological studies have shown seasonal variations in human reproductive capacity, which could be partially explained by variation of vitamin D concentrations throughout year.

In male infertility both low and high concentrations of vitamin D in serum negatively affect spermatozoa number, their progressive movement and morphology.

Although it is not yet established whether vitamin D truly has a role in human fertility, it is the theme worth of exploring.

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Diabetes mellitus and vitamin D

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Renewed interest in vitamin D, the so-called “sunshine vitamin,” has occurred recently because it has been linked to everything from cancer and heart disease to diabetes. However, most of the research is based on observational, epidemiological studies, which are important for generating hypotheses but do not prove causality.

Because destruction of β -cells usually begins in infancy or early childhood and continues until type 1 diabetes is diagnosed, it is intriguing in terms of the utility of vitamin D in people with type 1 diabetes. Currently, evidence supports that maintaining adequate vitamin D status during pregnancy, nursing, infancy, and childhood may help prevent type 1 diabetes. However, it is still unknown whether the genetics of type 1 diabetes place individuals at risk for vitamin D deficiency or whether vitamin D deficiency places individuals at risk for type 1 diabetes.

Some studies have suggested that low vitamin D concentrations might increase the odds of developing type 2 diabetes and that boosting concentrations could prevent. Newly published studies showed no likely connection between diabetes and vitamin D concentrations. Other studies findings suggest that high-dose supplementation of vitamin D can improve glucose metabolism to help prevent the development and progression of diabetes.

Adequate vitamin D supplementation may decrease the incidence of type 1 and possibly also of

type 2 diabetes mellitus and may improve the metabolic control in the diabetes state. However, the exact mechanisms and the level of protection are not clear and need further investigation.

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Vitamin D receptor polymorphisms in rheumatic diseases

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Vitamin D receptor (VDR) is responsible for biological actions of $1,25(\text{OH})_2\text{D}$. VDR is steroid, intracellular receptor, which consists of 427 amino acids located on chromosome 12. Dimerization of VDR with retinoic X receptor is responsible for activation of vitamin D target genes and their expression acting as transcription factor. Immunoregulatory properties of vitamin D in the cells of the immune system are mediated by VDR. Main effect of vitamin D in the immune system is downregulation of the Th1-driven autoimmunity. Low vitamin D concentrations in blood, may, among other factors, be associated with VDR polymorphisms by considering the potential immunosuppressive role of vitamin D in rheumatic diseases. Over 63 polymorphisms on VDR gene have been examined in rheumatic diseases for their association with disease development. Out of them, rs2228570, rs1544410, rs7975232, and rs731236 were the most common. Changes can be shown in non-coding parts of the gene (introns), and they then would not be seen in the protein product. These changes would then affect gene expression, and thus protein concentrations. Changes in the 5'-promoter of the VDR gene can affect mRNA expression patterns and concentrations, while 3' untranslated region (UTR) sequence variations can affect the mRNA stability and protein translation efficiency.

Furthermore, changes can take place in exonic parts of the DNA leading to changes in the protein sequence. However, various authors found heterogeneity in results. VDR polymorphisms may be the key to understand this heterogeneity. In understanding VDR gene polymorphism as a significant risk factor for rheumatic diseases, several facts need to be considered. Source of heterogeneity among studies might have been due to other factors, such as diversity in the population (age, ethnicity, sun exposure and dietary vitamin D intake, etc.), study design and genotyping methods. Some studies have shown that the ethnic (genetic) background, gene-gene or gene-environment interactions and lifestyle (sun exposure, dietary vitamin D intake, and obesity) might have a significant impact on increased risk of rheumatic diseases in association with polymorphisms.

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Vitamin D in lung diseases

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The role of vitamin D in regulating calcium homeostasis and bone metabolism is well known. However, vitamin D exerts pleiotropic protective functions including modulation of the adaptive and innate immune response. Its immunomodulatory function arises from: a) high affinity binding to the ubiquitously distributed vitamin D receptor (VDR) (including immune and pulmonary structural cells); b) the capacity of many cells and tissues to synthesize the active $1,25(\text{OH})_2\text{D}$ locally; and c) the ability of $1,25(\text{OH})_2\text{D}$ to control the transcription of various genes associated with multiple biological

processes through nuclear VDR binding. Alterations in these metabolic pathways together with high prevalence of vitamin D insufficiency have been linked to chronic lung diseases (e.g. chronic obstructive pulmonary disease (COPD), asthma, respiratory infections etc.).

The prevalence of vitamin D deficiency is high in COPD patients, positively correlates with disease severity and is strongly associated with pulmonary function and underlying osteoporosis in COPD patients. Reduced vitamin D concentrations are thought to enhance pro-inflammatory pathways and reduce immunity in these patients, contributing to disease deterioration. Vitamin D supplementation in COPD patients reduces morbidity by reducing the risk of hip fractures, and might prevent deterioration of pulmonary function.

It was proposed that vitamin D deficiency found in asthma patients impairs lung function, promotes airway hyperresponsiveness and inflammation, and decreases response to glucocorticoids. Animal and *in vitro* studies support these hypotheses. Epidemiologic and *in vivo* studies found an association between low serum vitamin D and decline in lung function in patients with asthma. However, results from clinical trials are conflicting - it is still unclear whether vitamin D supplementation offers a valid treatment option in asthma patients.

Decreased serum vitamin D is associated with increased susceptibility to respiratory tract infections (*i.e.* tuberculosis and influenza). However, insufficient evidence is available to define its role in prevention and/or treatment of tuberculosis and viral upper respiratory tract infections.

Larger trials with longer follow-up are needed to elucidate the molecular mechanisms linking vitamin D to chronic lung diseases, as well as the therapeutic effects of vitamin D supplementation.

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