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“Assessment of vitamin D status in target groups by selective chromatographic method for 25-hydroxy vitamin D”

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Recently, many data has been accumulated on the role of vitamin D not only in relation to bone-mineral metabolism, but also on its ability to reduce the risk of a number of chronic diseases such as chronic renal failure, diabetes, cardiovascular diseases, malignant diseases, multiple sclerosis, and diseases with disorders in cognitive functions. Two main forms of vitamin D are found in the human body: vitamin D_3 (cholecalciferol) and its plant counterpart vitamin D_2 (ergocalciferol). Once in the body, it is metabolized to 25-hydroxy vitamin D_3, (25-OH D_3) and to 1,25-dihydroxy vitamin D_3 (1,25-$(OH)_2$ D_3 or calcitriol). The metabolically active form is calcitriol, performing an endocrine and autocrine function. A barometer for vitamin D status in the body is its precursor 25-OH D_3 – the main circulating form of vitamin D in the blood.

The increase in the number of cases of vitamin D deficiency in recent years worldwide has acquired an alarming trend. This necessitates the monitoring of vitamin D levels both in healthy individuals in different age groups (children, pregnant women and the elderly) and in certain risk groups of patients – with osteoporosis, with chronic kidney diseases, transplants, diabetes, hypertension, with autoimmune and oncological diseases and obese individuals. The children’s group is particularly important, due to possibilities for prevention and prevention of the occurrence of chronic diseases at a later age. All this makes it particularly imperative to introduce a reliable analytical method for determining vitamin D status and establishing plasma cut-off values for different physiological and pathological conditions, age groups and their seasonal variations.

There are currently two main analytical approaches for the quantification of 25-OH D_3: immunochemical and chromatographic. Immunochemical methods are more widespread, due to their easier execution, but their main disadvantages are their inability to selectively determine 25-OH D_3 and 25-OH D_2, detected when supplementing with vitamin D_2, and the lesser extent of their reproducibility and accuracy. Chromatographic methods have higher analytical reliability; they can selectively determine 25-OH D_3 and 25-OH D_2 as well as the C3-epimer, important in assessing vitamin D status in infancy. Their disadvantages are higher labor intensity, more expensive equipment, as well as the need for highly qualified personnel. In recent years, Europe and the USA have worked hard to standardize methods for the quantitative analysis of 25-OH D. Expert committees have reached the consensus opinion that chromatographic methods should be used as references. In Bulgaria, no reliable chromatographic method has yet been developed, and the determination of 25-hydroxy

vitamin D_3 levels is carried out by immunochemical methods in a limited number of laboratories in the country. These facts justified the idea of the present pilot study to develop a chromatographic method with high analytical reliability for the assessment of vitamin D status.

As a logical conclusion of the work on the introduction of the method will be its application to assess the vitamin D status in certain target groups: patients with chronic renal failure and healthy pre-pubertal children with normal and overweight weight.

Chronic kidney disease (CKD) is characterized by low levels of the active form of vitamin D_3, even in the early stages of the disease, when there are still no changes in the serum levels of calcium, phosphorus and parathormone. Studies from recent years have established a positive correlation between the deficiency of 25-OH D_3 and the occurrence and severity of the anemic syndrome in patients with CKD. Clarifying the dynamics of plasma 25-OH D_3 changes in CKD patients at different stages of the disease, as well as its interrelationships with parameters assessing calcium metabolism and erythropoiesis, will be a prerequisite for adequate therapeutic behavior, including vitamin D supplementation, its analogues and calcium mimetics.

Through its non-calcitropic effects, vitamin D regulates the function of many organs and systems, including the endocrine pancreas, liver and adipose tissue. Subclinical vitamin D deficiency has been suggested to be a risk factor for insulin resistance, beta-cell dysfunction, type 1 and 2 diabetes, and metabolic syndrome. As a result, vitamin D deficiency leads to secondary hyperparathyroidism with a subsequent increase in intracellular calcium in adipocytes, stimulating lipogenesis and obesity. A vicious cycle of metabolic events ensues: the increase in adipose tissue is a prerequisite for vitamin D sequestration in fat depots, and the resulting reduced vitamin D bioavailability and subclinical deficiency lead to increased lipogenesis and obesity. On the other hand, even the relatively small number of studies on vitamin D status in children show an alarming trend of increasing cases of vitamin D deficiency/ insufficiency. This is a reason to study vitamin D status in normal weight and obese children and its seasonal variation. Establishing vitamin D deficiency in children and the possibility of supplementing them with vitamin D preparations would support the strategic health goal of reducing the risk of developing various chronic diseases in adulthood, in the pathogenesis of which the endocrine axis of vitamin D is involved.

In conclusion, there is a lack of information on the frequency of vitamin D deficiency and insufficiency in Bulgaria. Bearing in mind the published data for other European countries and the USA, showing that vitamin D deficiency reaches epidemic proportions, it becomes imperative to make efforts in this direction in Bulgaria as well. This pilot study may set the stage for future large-scale research.

Purpose of the study:

Development and validation of a selective liquid chromatographic method for monitoring plasma levels of 25-hydroxy vitamin D_3 and its application for assessment of vitamin D status in patients with chronic kidney disease and healthy prepubescent children with normal and overweight weight.

Scientific idea or working hypothesis (formulation):

In the last decade, the global scientific community has paid great attention to the role of vitamin D in maintaining optimal health. The discovery of its non-calcemic effects led to an update of the norms for average daily intake and, accordingly, to the need to monitor its level in the blood. The study of the circulating form of vitamin D has entered widely into the clinical practice of European countries and the United States. In this regard, Bulgaria lags significantly behind. Testing for vitamin D status is performed extremely rarely in a limited number of laboratories and with less analytically reliable methods. This prompted us to develop and introduce a reliable chromatographic method for monitoring vitamin D status.

The current pilot study will be conducted in two stages:

1. Analytical stage: Development, validation and testing of a direct and selective liquid chromatographic method with UV detection for measuring plasma levels of 25-hydroxy vitamin D_3 and 25-hydroxy vitamin D_2. The reliability of the developed method will be evaluated by comparison with proposals from international organizations as a reference liquid chromatographic method with mass spectrometric detection, as well as by including it in the international program for external quality assessment of the results of the analyzes of 25-OH D in serum (DEQAS Vitamin D External Quality Assessment Scheme). The obtained results will be compared with those of the currently widely used immunochemical methods in the clinical laboratory.
2. Clinical stage: The monitoring of the 25-OH D levels is imperative to be carried out both in healthy individuals for prophylactic purposes and in risk groups of patients. In the present study, the vitamin D status will be investigated in two target groups of the Bulgarian population:
   - ❖ Patients with chronic kidney disease (CKD), in which treatment also includes supplementation with vitamin D_3 preparations and its analogs. Correlations with disease stage and type of therapy will be sought. The effect of supplemental therapy with vitamin D preparations and analogs will be evaluated;
   - ❖ Normal-weight and obese prepubescent children in whom vitamin D status and its seasonal variations will be assessed. The frequency of vitamin D deficiency and insufficiency will be established with a view to the prevention of chronic diseases in adulthood. Correlations between vitamin D status and lifestyle, diet and anthropometric parameters will be sought.

The clinical stage will begin after obtaining permission from the Research Ethics Committee at the MU-Varna.

Research methods used:

1. Methods:
   - ❖ High-performance liquid chromatography with detection in the ultraviolet and visible region of the spectrum for the determination of 25-OH D for assessment of vitamin D status;
   - ❖ Routine clinical-laboratory methods for assessing bone-mineral exchange;
   - ❖ Anthropometric measurements: body weight (with a digital scale, accurate to 0.1 kg); height (with a stadiometer accurate to 1 cm); waist circumference (accurate to 1 mm); BMI; assessment of prepubescent development (by secondary sex characteristics according to the Tanner criteria);
   - ❖ Statistical methods for analyzing the results.
2. Patients:
   - ❖ Patients with chronic renal failure – without renal function replacement therapy and receiving renal function replacement therapy (dialysis and peritoneal dialysis) and renal transplant recipients;
   - ❖ A representative sample of normal-weight and obese prepubescent children.

Characteristics of the expected contribution (practical or theoretical orientation):

Expected theoretical contribution:

1. Data on the vitamin D status of healthy pre-pubescent children from the Bulgarian population will also be obtained.
2. Additional data on the bioavailability of 25-OH D_3 in obese children, which are scarce in the world literature, will be obtained.

Expected practical contribution:

1. At the moment, there is no accurate and selective chromatographic method developed in Bulgaria for the determination of 25-hydroxy D_3/D_2 in biological environments. The development and validation of such a method will allow monitoring of 25-hydroxy D_3 in blood serum of both healthy individuals and risk groups of patients;
2. The availability of a working method with a high degree of analytical reliability will make it possible to assess the vitamin D status in a risk group of patients and in healthy children with normal weight and obesity. This will allow in the future to define reference ranges for serum levels of 25-hydroxy D_3, which are currently undefined;
3. Accurate quantitative assessment of vitamin D-status will allow to optimize some aspects of the therapy of the underlying disease in patients with CKD and to make decisions about supplementation with vitamin D_3/D_2 and analogues;

4. Determining the vitamin D status in healthy children with normal weight and obesity will justify the need for supplementation in case of marked vitamin D deficiency/ insufficiency and prevent the possible risk of developing chronic diseases in adulthood;
5. Introduction of a laboratory method that can also be used in routine clinical practice on the territory of our region.

Expected contribution of an educational and training nature:

1. The study also included young scientists (under 35), as well as medical and pharmacy students. As participants in the research team, they will acquire practical knowledge and skills in the course of method development and verification, in the collection of samples, analysis of the obtained results and their preparation for publication;
2. The proposed study will serve as a basis for the development of a dissertation work in the field of clinical laboratory.