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Factor analysis of clinical and biochemical parameters of bone remodeling changes associated with leading VDR polymorphisms in patients with aseptic necrosis of the femoral head

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Abstract

Introduction Bone metabolic markers informatively reflect the complex balance of bone formation/resorption and are widely used in clinical practice. Genetic predisposition also plays a significant role in the etiopathogenesis of aseptic necrosis of the femoral head (ANFH). We **aimed** to establish probable associations between a number of biochemical parameters of bone tissue metabolism with clinical ones (sex, age, body mass index (BMI)), disease stage, T and Z criteria) with regard to the leading polymorphisms of the vitamin D_3 receptor gene (*VDR*) in patients with ANFH. **Materials and Methods** Based on clinical and biochemical examination of 273 patients with ANFH, factor analysis was performed. **Results** Clinical parameters (age, BMI) correlated most with biochemical parameters. Age showed most association with β-CrossLaps, DPD, osteocalcin, and osteoprotegerin; BMI, in turn, was associated with $1.25(OH)_2D$, 25(OH)D, Ca, and DPID. Carriers of G/G genotype A-3731G were found to have more than a three-fold increased risk of ANFH compared to healthy controls; carriers of G allele had a 2.5-fold increased risk. Carriage A/A genotype +283 A > G increases the risk of developing ANFH by 2.4 times. Carriage of the A allele of the same locus is associated with a 1.5-fold increased risk of ANFH. **Discussion** We determined a reliable association of age with β-CrossLaps, DPD, osteocalcin, osteoprotegerin, and BMI with $1.25(OH)_2D$, 25(OH)D, Ca, and DPD in patients with ANFH, suggesting significant prospects for using these biochemical parameters to monitor changes in bone tissue remodeling. An increased risk of ANFH was established in the presence of the G/G genotype and the G allele of the A-3731G (Cdx2) locus, the A/A genotype of the +283 A > G (BsmI) locus in the *VDR* gene. **Conclusion** Associations of BMI with $1.25(OH)_2D$, 25(OH)D, Ca, DPD characterize the relationship between resorption and osteogenesis processes and somatic parameters of the examined patients with ANFH. The genotypic variants G/G rs11568820 and A/A rs1544410 in the *VDR* gene are associated with an increased risk of AFHD.
Keywords: aseptic necrosis of the femoral head, biochemical markers of bone tissue metabolism, vitamin D receptor gene, *VDR*

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INTRODUCTION

Avascular necrosis of the femoral head (ANFH) is a severe, irreversible disease associated with alcohol, steroids, trauma, or unclear (idiopathic) etiology, affecting mainly the middle-aged population [1]. In this pathology, a part of the femoral head becomes necrotic, which is most often associated with ischemia [2] due to impaired arterial blood supply and venous outflow. In traumatic etiology of ANHF, arterial blood flow is interrupted, which is the main cause of ischemia. Although such a lesion can occur in any bone of the skeleton, it is the femoral head that is most frequently affected, which is associated with a systematic axial load and the characteristics of its blood supply [3]. In the absence of specialized medical care, the end result of ANFH is the destruction of the necrotic femur and articular cartilage, which leads to severe secondary arthrosis in approximately 60-70 % of patients [4].

In radiation-induced, alcohol- or steroid-associated ANHF, which are not embolic infarctions, a kind of intraosseous compartment syndrome occurs. ANFH is a multifactorial disease with an important component of genetic predisposition when exposed to risk factors [5, 6]. Genetic predisposition plays a significant role in the development of ANFH in some people who use steroids and abuse alcohol [7]. Due to their ability to specifically and dynamically reflect the mechanisms of bone turnover within the complex balance between its formation and resorption, biochemical markers of bone turnover have become widely used in clinical practice [8, 9].

An important role in the development of ANFH is played by the state of the structure and mineral density of the bone, since the risk of necrotization increases significantly in ischemia and reduced mineral density. Mineral metabolism in the human body depends not only on the content of calcitriol, but also on the sensitivity of target organs to it [10], which is genetically determined. A well-known role in bone metabolism is the vitamin D receptor (encoded by the *VDR* gene), a nuclear receptor that binds to DNA and acts as a transcription factor. At the same time, different parts of the receptor have specific functions, which include DNA binding, receptor dimerization, and activation of genes and cofactors. The best known *VDR* gene polymorphisms are BsmI (rs1544410), TaqI (rs731236), ApaI (rs7975232), FokI (rs2228570) and Cdx2 (rs11568820). These allelic variants are associated with certain clinical outcomes due to their effect on receptor structure or mRNA stability. The VDR Cdx2 polymorphism is located in the promoter region of the VDR gene and is believed to be associated with the degree of calcium absorption in the intestine and activation of the vitamin D receptor. The G allele reduces the transcriptional activity of VDR compared to the A allele by 70 %, which has an effect on calcium absorption, mutant form – alleles A – are characterized by resistance to the loss of mineral components of bone tissue with a lack of exogenous calcium [11, 12]. *VDR* BsmI is located in the 3'-untranslated region and is involved in the regulation of mRNA stability of the *VDR* gene [13]. Compared with carriers of the GG genotype, carriers of the AA genotype have an increased risk of developing postmenopausal osteoporosis [13] and femoral fractures [14].

The study of etiopathogenesis and improvement of diagnostic algorithms for aseptic necrosis of the femoral head are topical issues of restorative orthopedics.

The **aim** of the work was to establish possible associations of a number of biochemical parameters of bone tissue metabolism with clinical ones (gender, age, body mass index (BMI), disease staging, T and Z-criteria), considering the leading *VDR* polymorphisms in patients with ANFH.

MATERIAL AND METHODS

At the laboratory of Hemotest Ltd (Moscow) in the blood serum of patients, the concentration of total calcium was determined (by photometry); HPLC (Agilent 1200, Makron, Germany) was used to study the content of vitamin K, active forms of vitamin D (1.25(OH)$_2$D and 25(OH)D); levels of osteoprotegerin, parathyroid hormone, osteocalcin, C-terminal telopeptides of type I collagen (β-Cross laps) using electrochemiluminescence (Cobas 8000, Roche, Germany); deoxypyridinoline (DPD) in urine was determined by the chemiluminescent method (Immulite 2000, Siemens, USA). Isolation of total DNA from venous blood leukocytes was performed using a Thermo Scientific KingFisher Flex King magnetic particle processor. VDR single nucleotide polymorphisms were determined by PCR (Eppendorf MasterCycler Nexus Gradien, Germany) and pyrosequencing (Pyro Mark Q24 QIAGEN, Germany). PCR was performed using reagent kits from Isogen Laboratory Ltd and allele-specific oligonucleotides produced by Evrogen CJSC.

The clinical and biochemical study included 273 people (162 women and 111 men) aged 18-92 years, the average age of women was 60.6 ± 1.1 years, and 54.0 ± 1.4 years of men, with a verified diagnosis of ANFH of different grades according to the ARCO classification. Using the HarrisHip score questionnaires, the functional assessment of the joints was determined. Ultrasonic sonography (Sunlight MiniOmni, Israel) was used to determine densitometric parameters on the proximal phalanx of the third finger of the non-dominant upper limb and radius (T and Z criteria). According to the indications, magnetic resonance and computed tomography studies were performed along with X-ray study of the hip joint (three projections: prone, supine, Launstein abduction).

Statistical analysis of clinical and laboratory data was performed using Epiinfo™ (http://www.cdc.gov/epiinfo/), WinPepi (http://www.brixtonhealth.com/pepi4windows.html) and a specialized calculator (https://calc.pcr24.ru/index.php). To identify possible associations of biochemical indicators of bone tissue remodeling with clinical indicators and to identify the leading factors that determine the variability of clinical and biochemical parameters, we used the algorithm of factor analysis of principal components with Varimax-rotation. The selection of the main factors in the calculation procedure is based on the calculation of the Pearson correlation coefficients between the initial parameters, including clinical ones – age, gender, disease stage ARCO, BMI, T- and Z-criteria – and biochemical ones – vitamin K, 25(OH)D, 1, 25(OH)$_2$D, osteocalcin, osteoprotegerin, parathyroid hormone, Ca, DPID, β-Cross laps. Significant factors were identified taking into account the factor load exceeding 1.

For analyzing the frequencies of polymorphic markers, we studied the correspondence of the studied and control samples to the fulfillment of the Hardy-Weinberg equilibrium conditions with the calculation of χ^2 and Fisher's exact test. The degree of association of the genotype and alleles with the disease was assessed by the odds ratio (OR) [14] taking into account the 95 % confidence interval (95 % CI) using multiplicative and additive models of inheritance. All patients were residents of the Central Federal District and were homogeneous in terms of ethnogenetic affiliation: they belonged to the Slavic-Baltic group.

RESULTS

The factor analysis identified 6 main factors that explain the largest portion of variability of 14 variables with a total variance of 73.68 %. The indicators of the relationship between clinical parameters and biochemical parameters of remodeling in patients with ANFH according to the results of factor analysis are presented in Table 1.

Table 1

Factor Loads of Clinical Parameters and Biochemical Indices of Remodeling in Patients with ANFH

Parameter	Factor					
	1	2	3	4	5	6
Age	-0.453	-0.307				0.519
BMI			-0.522		0.473	
Gender	-0.774					
Stage (ARCO)				0.825		
Criterion T	0.909					
Criterion Z	0.820					
β-CrossLaps		0.864				
Ca					0.756	
DPD		0.624		0.302	-0.334	
$1.25(OH)_2D$			0.925			
25(OH)D			0.919			
Osteocalcin		0.922				
Osteoprotegerin						0.869
Parathyroid hormone				0.548		
Vitamin K					-0.593	
Isolated dispersions (%)	18.89	16.42	13.40	9.48	8.60	6.88

Note: factor loads of indicators with a value of less than 0.3 are not shown

The procedure for ranking biochemical parameters according to the frequency of associations (association with three parameters) with clinical ones enabled to identify the priority clinical and laboratory indicator DPD, excreted in the urine. Similarly, when ranking associations of already clinical characteristics with biochemical parameters, priority ones were identified as: age, BMI. Based on the results of factor analysis, age is associated with such biochemical parameters as β-CrossLaps, DPD, osteocalcin, osteoprotegerin. BMI is characterized by associations with such biochemical parameters as levels of $1.25(OH)_2D$, 25(OH)D, Ca, DPD.

Analyzing the factor loads of the six identified factors, the first factor with the highest factor load (1.889) is probably associated with age-related changes in bone tissue characteristics (mineral density), based on strong direct correlations of densitometry parameters (T-test – 0.909 and Z -criterion - 0.820), a strong feedback with gender (-0.774) and a weak relationship with age (-0.453). The second selected factor (factor load 1.642), apparently, reflects the ratio of the processes of bone tissue metabolism during its formation and resorption, since it has strong direct correlations with markers of osteogenesis (osteocalcin – 0.922) and resorption (β-CrossLaps – 0.864) and correlation relationship of average strength with the level of DPD in urine (0.624). The third component (factor load 1.340) is characterized by the presence of strong direct correlations between blood levels of $1.25(OH)_2D$ (0.925) and 25(OH)D (0.919) and an inverse correlation of medium strength (-0.522) with such an indicator as BMI. This factor, in our opinion, can characterize the relationship between osteogenesis processes and somatic parameters. The fourth factor with a load of 0.948 is characterized by a weak correlation with the content of DPD in the urine (0.302) against a strong correlation (0.825) with such an increase in the severity of the pathological process as the ARCO stage and a positive correlation with the concentration of parathyroid hormone (0.548). This factor, apparently, characterizes the severity of the pathological process in the bone tissue with the predominance of resorption processes over the processes of bone tissue remodeling against an increase in parathyroid hormone levels. The fifth factor (0.860) is defined by us as an aging factor, since it includes a strong positive association with the concentration of total Ca in the blood (0.756), a medium-strength correlation with the clinical BMI (+0.473), which is similar in strength to the negative with the level of vitamin K (-0.593) and slightly negative with excreted DPD (-0.334). In this case, there is a trend of increasing BMI in combination with age-related resorption (loss of calcium in bone tissue – an increase in calcium levels in the blood) against a decrease in vitamin K (which contributes to demineralization) and excretion of DPD (a marker of resorption of the organic matrix of bone tissue). The sixth factor with the value of the selected variance of 0.688 is characterized by a high association with the level of osteoprotegerin (0.869) and a weak association with such a clinical sign as age (0.519). In the examined patients, there is a tendency for some increase with age in the level of osteoclastogenesis inhibitor – osteoprotegerin, which may be due to the effect of therapeutic measures that were carried out in patients.

The analysis of the data given in Table 2 found that in patients with a diagnosed ANFH, the frequency of carriage of GG, AG and AA genotype variants in the A-3731G (Cdx2) polymorphic locus of *VDR* (rs11568820) was

72.5, 25.3, 2.2 % did not statistically significantly coincide with population frequencies – 44.8, 49.2 and 6.0 % ($\chi^2 = 37.35$; df [0, 1, 2], 1, P = 3.0 E-9) [15]. For carriers of G/G genotypic variant, there is more than a threefold increase in the likelihood of this pathology (OR=3.25: CI 2.26-4.68). Among patients diagnosed with ANFH, the proportion of carriers of the G allele was 0.852 (85.2 %) and statistically significantly exceeded the proportion of carriers of this allelic variant in the control group (69.4 %) (F = 0.000024, $\xi^2 = 17.84$). In persons with G allele carriers, there is a 2.5-fold increase in the risk of developing ANHF (OR = 2.53, CI1.87-3.43).

Subsequent analysis of the data revealed a statistically significant difference in the genotypic frequencies of the GG, AG, and AA locus +283 G/A (BsmI) between the groups of patients with ANHF and the population sample (Table 2): 41.4, 42.9, 15.8 % and 7.3, 41.1, 51.6 respectively ($\chi^2 = 6.75$; df [0, 1, 2], 1, P = 0.03); carriage of the A/A genotype of the +283 A > G (BsmI) locus contributes to a 2.4-fold increase in the risk of developing this pathology (OR = 2.39; 95 % CI: 1.13-5.07). Carriers of the A allele of this locus were found to have an increased risk of developing ANFH (OR = 1.54; 95 % CI: 0.11 2.13). The percentage of carriers of the A allele of this locus in patients with ANFH was 37.2 %, which is statistically significantly higher than in the population group (27.8 %) (F = 0.05, $\xi^2 = 3.74$).

It should be noted that the analysis revealed no significant differences in clinical and biochemical parameters in carriers of different genotypes. Also, there were no statistically significant differences in the distribution of genotypes between groups of patients with normal or low levels of vitamin D.

Table 2

Features of the distribution of allelic and genotypic frequencies of *VDR* polymorphic loci among examined patients with ANHF and healthy population controls

Genes and polymorphisms	Genotypes and alleles	Genotype rate, %		OR (95 % CI)	P
		Patients with ANFH	General population sample		
A-3731G (Cdx2) (rs11568820)*	GG. n (%)	198 (72.5)	112 (44.8)	3.25 (2.26–4.68)	3,0 E-9
	AG. n (%)	69 (25.3)	123 (49.2)	0.35 (0.24–0.50)	
	AA. n (%)	6 (2.2)	15 (6.0)	0.32 (0.13–0.92)	
	G. n (%)	233 (85.2)	173 (69.4)	2.53 (1.87–3.43)	2,4 E-5
	A. n (%)	40 (14.8)	51 (41.1)	0.40 (0.29–0.53)	
+283 A > G (BsmI) (rs1544410)**	GG. n (%)	113 (41.4)	64 (51.6)	0.66 (0.43–1.01)	0,03
	AG. n (%)	117 (42.9)	51 (41.1)	1.07 (0.70–1.65)	
	AA. n (%)	43 (15.7)	9 (7.3)	2.39 (1.13–5.07)	
	G. n (%)	171 (62.8)	90 (72.2)	0.65 (0.47–0.90)	0,05
	A. n (%)	102 (37.2)	34 (27.8)	1.54 (0.11–2.13)	

Notes: * – allelic and genotypic frequencies are given from the publication [16]; ** – similar frequencies are given from the source [17].

DISCUSSION

The analysis of the study data revealed a clinical and laboratory indicator that is a priority in ANFH. It is DPD, excreted in the urine, which indicates an increase in resorption processes in bone tissue in ANFH. Collagen fibrils include many cross-linked amino acids that effectively stabilize the formed collagen molecule. These include pyridinoline (PYD), a cross-linked polymer formed from three hydroxylysine residues, and deoxypyridinoline (DPD), which is formed from two hydroxylysine residues and one lysine residue. In the collagen of most tissues, the PYD/DPD ratio is 10:1, while in bone tissue it is 3-3.5:1. Moreover, the level of DPD correlates well with bone turnover. An additional characteristic that makes evaluation of pyridine crosslinks ideal is that they are not metabolized upon release and are not absorbed from food. They are excreted in the urine in free (40 %) and peptide-bound (60 %) forms. Since cross-link molecules are found only in mature collagen, their increased excretion in the urine reflects the intensity of degradation of mature collagen and is not a clear indicator of newly synthesized bone collagen and, thus, indicates active bone resorption [18, 19].

An increase in the level of DPD was noted in patients with osteonecrosis of the femoral head compared with healthy controls [20]. Based on the results of factor analysis, we established a similar trend: the presence of associations of such a clinical indicator as age with clinical and laboratory indicators of bone tissue metabolism – markers of β-CrossLaps and DPD resorption [21]. Associations of this clinical parameter with osteogenesis markers, osteocalcin and osteoprotegerin, were also revealed [22-24]. An increase in the level of osteocalcin and osteoprotegerin in the examined patients with ANFH may be due to the effect of the therapeutic measures taken by the patients.

Age, trauma, hormonal disorders, mutations in osteoregulatory genes, vitamin D deficiency, and other factors are associated with the formation of bone tissue pathology [25]. The interaction of these factors increases the likelihood of developing these diseases [26]. In a Japanese population-based study, the age-adjusted incidence of ANHF was found to be 2.51 cases per 100,000 person-years. This pathology occurs 1.6 times more often in men [27]. The risk factors for the development of ANFH also include the use of tobacco, alcohol, and treatment with steroid drugs [28, 29].

A 2015 retrospective study of more than 30,000 Chinese adults found that 0.725 % of the individuals were diagnosed with ANFH. Male gender, hypercholesterolemia, urban living, family history, smoking, alcohol, long-term treatment with glucocorticoids and obesity were referred to significant risk factors. [30]. At the same time, other researchers did not find any association between the supposed risk factors for the development of ANFH, such as alcohol consumption, steroid use, age, BMI, and gender [31]. Older people, especially postmenopausal women, are at higher risk of developing the disease due to estrogen deficiency [32].

In the course of this study, associations of such a clinical parameter as BMI with $1.25(OH)_2D$, 25(OH) D, Ca, DPD were established, which can characterize the relationship between the processes of resorption-osteogenesis and the somatic parameters of the examined patients with ANFH.

In this study, we studied the influence of two variants of the *VDR* gene (rs11568820 and rs1544410) as a genetic determinant of ANFH among residents of the Central Federal District. All patients were homogeneous by ethnogenetic affiliation: they belonged to the Slavic-Baltic group. The results obtained show that the G/G rs11568820 and A/A rs1544410 genotypic variants in the *VDR* gene are significantly associated with an increased risk of AFHD. According to the literature, these genotypes are associated with increased bone mass loss [33-39]. It has been suggested that these polymorphisms affect bone mineral density through direct regulation of receptor activity. Structural and unctional changes in *VDR* molecules lead to a decrease in the activity of vitamin D receptors and a decrease in Ca absorption in the intestine [40].

CONCLUSION

Ranking of the biochemical parameters of patients with ANFH showed that DPD, excreted in the urine, takes the first place in terms of the frequency of associations with clinical indicators. The ranking of clinical characteristics by association with biochemical parameters enabled to identify the most significant of them: age and BMI. Age was most associated with the following biochemical parameters: β-CrossLaps, DPD, osteocalcin, osteoprotegerin. BMI, in turn, is associated with such parameters as levels of $1.25(OH)_2D$, 25(OH D, Ca, DPD. In the course of the factor analysis, a number of key biochemical parameters were established that are in close relationship with the main clinical indicators (age, BMI) of ANFH.

The results of the study showed that the carriage of the genotypic variant G/G A-3731G (Cdx2) *VDR* gene contributes to a 3.25-fold increase in the probability of developing ANHF (OR = 3.25: CI2.26-4.68). The rate of G allele carriers in the group of patients diagnosed with ANHF was 85.2 % and statistically significantly exceeded that of the population group (69.4 %) ($F = 0.000024$, $\xi^2 = 17.84$). In persons with G allele carriage, the probability of developing AFNF is increased by 2.5 times (OR = 2.53, CI1.87-3.43). The genotypic frequencies of the GG, AG, and AA locus +283 G/A (BsmI) *VDR* in the group of patients with ANFH and the control sample significantly differ: 41.4, 42.9, 15.8 % and 7.3, 41.1, 51.6 respectively ($\chi^2 = 6.75$; df [0, 1, 2], 1, $p = 0.03$); with the carriage of the A/A genotype of this locus, the risk of developing ANHF is increased (2.4 times) (OR = 2.39; 95 % CI: 1.13-5.07). Carriers of the A allele of this locus have an increased risk of developing ANFH (OR = 1.54; 95 % CI: 0.11-2.13); among patients with ANFH, the rate of carriage of the A allele in this locus significantly exceeded that of the control group, 37.2 % and 27.8 %, respectively ($F = 0.05$, $\xi^2 = 3.74$).

The use of key biochemical parameters that are closely related to the main clinical characteristics of ANFH enables to optimally monitor changes in bone tissue remodeling in regard to hereditary predisposition and, therefore, contribute to the provision of effective orthopedic care.

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