

CLINICAL LABORATORY DIAGNOSTICS

A SPECTROPHOTOMETRIC METHOD FOR DETERMINING THE PHENOTYPE OF ACETYLATION BASED ON CAFFEINE METABOLISM

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RESUME

Justification. The multiplicity of diseases and conditions in which NAT2 plays a key role, dictates the need to study both the genotype and the phenotype of acetylation. Such approaches will make it possible to identify risk groups, as well as select effective doses of drugs, preventing the development of side effects and determining the optimal therapeutic dose.

The aim. To develop an economical and simple method for determining the phenotype of acetylation based on the spectrophotometric determination of the metabolic rate of caffeine as a test preparation.

Methods. The work was performed in a group of 52 patients with spinal canal stenosis (average age 51.04 ± 9.671 years) and 32 conditionally healthy people (average age 53.32 ± 0.933 years). Creatinine and uric acid were determined in blood and urine samples before and after taking caffeine, and the optical density of urine at 272 nm was measured. To estimate the acetylation rate, the calculation of an integral predictive indicator is proposed. PCR with specific primers was used as a reference method to detect NAT2 polymorphisms affecting the acetylation rate.

Results. An effective, easily executable algorithm has been developed that includes the technology of collecting, preparing and analyzing blood and urine samples before and after using caffeine as a test drug, and calculating an integral predictive indicator to assess the rate of acetylation reactions. The method is suitable for indirect, simpler and less costly assessment of the rate of acetylation reactions compared with molecular genetic studies.

Conclusion. The determination of the acetylation phenotype using the proposed analytical procedures makes it possible to identify individual differences in connective tissue metabolism, and to seek approaches to predicting treatment outcomes depending on the polymorphic variant of NAT2 with the identification of risk groups. The data obtained can be used to search for new links in pathogenesis, to implement the mechanisms of pain and fibrosis.

Keywords: acetyltransferase NAT2, acetylation, prediction, caffeine, uric acid, creatinine

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РАЗРАБОТКА СПЕКТРОФОТОМЕТРИЧЕСКОГО МЕТОДА ОПРЕДЕЛЕНИЯ ФЕНОТИПА АЦЕТИЛИРОВАНИЯ НА ОСНОВЕ МЕТАБОЛИЗМА КОФЕИНА

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РЕЗЮМЕ

Обоснование. Многочисленность заболеваний и состояний, при которых ключевую роль имеет NAT2, диктует необходимость исследования как генотипа, так и фенотипа ацетилирования. Такие подходы позволят определять группы риска, а также подбирать эффективные дозы лекарств, предотвращая развитие побочных эффектов и определяя оптимальную терапевтическую дозу.

Цель. Разработать экономичный и простой метод определения фенотипа ацетилирования на основе спектрофотометрического определения скорости метаболизма кофеина в качестве тестового препарата.

Методы. Работа проведена в группе из 52 пациентов со стенозами позвоночного канала (средний возраст $51,04 \pm 9,671$ лет) и 32 условно здоровыми людьми (средний возраст $53,32 \pm 0,933$ лет). В образцах крови и мочи до и после приема кофеина определяли креатинин и мочевую кислоту, а также измеряли оптическую плотность мочи на 272 нм. Для оценки скорости ацетилирования предложен расчет интегрального прогностического показателя. В качестве референсного метода использовали ПЦР со специфическими праймерами для выявления полиморфизмов NAT2, отражающихся на скорости ацетилирования.

Результаты. Разработан эффективный, легко выполнимый алгоритм, включающий технологию сбора, подготовки и анализа образцов крови и мочи до и после применения кофеина в качестве тестового препарата, расчет интегрального прогностического показателя для оценки скорости реакций ацетилирования. Метод пригоден для косвенной, более простой и мало затратной оценки скорости протекания реакций ацетилирования по сравнению с молекулярно-генетическими исследованиями.

Заключение. Определение фенотипа ацетилирования с помощью предлагаемых аналитических процедур позволяет выявить индивидуальные различия в метаболизме соединительной ткани, искать подходы к прогнозированию исходов лечения в зависимости от полиморфного варианта NAT2 с выделением групп риска. Полученные данные можно использовать для поиска новых звеньев патогенеза, реализации механизмов боли и фиброобразования.

Ключевые слова: ацетилтрансфераза NAT2, ацетилирование, прогнозирование, кофеин, мочевая кислота, креатинин

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INTRODUCTION

Genetic polymorphism is known to exist in N-acetyltransferase 2 (*NAT2*), which results in the formation of either fast or slow acetylating phenotypes. The primary metabolic pathway for the substrates of this enzyme, heterocyclic amines, involves N-hydroxylation in the liver through the action of CYP1A2, followed by O-acetylation via *NAT2* in the second phase [1].

Decreased acetylation activity typically occurs due to reduced protein synthesis, including enzymes involved in this process [2], formation of acetyltransferase variants with lower enzymatic activity and/or stability [3], or enhanced degradation of acetyltransferases [4]. The most frequent cause of altered *NAT2* activity is genetic polymorphism, which is associated with the presence of one or more single nucleotide substitutions in the gene [3, 4].

The acetylation status has been shown to influence both the frequency and severity of various diseases, including oncological diseases [5, 6, 7]. It may also be linked to insulin resistance, lipid metabolism abnormalities [8], type 2 diabetes [9], pancreatitis [10], allergies [11], systemic lupus erythematosus [12], rheumatoid arthritis, psoriasis, Parkinson's disease [13], and other conditions.

Slow *NAT2* acetylation status is typically associated with an increased risk of adverse pharmacotherapy effects, especially hepatotoxicity. Conversely, individuals with fast acetylation are more likely to experience treatment failure due to rapid drug elimination and subsequent disease relapse [14]. Arylamine N-acetyltransferases *NAT1* and *NAT2* are primarily involved in the second phase of detoxification, but they are also expressed in various organs and tissues, potentially performing other physiological functions. Apart from hepatocytes, these enzymes can be found in leukocytes, fibroblasts, mononuclear cells, the urothelium, intestines, kidneys, bronchial epithelia, periodontal tissues, human skin, prostate, and other locations [15, 16].

The role of *NAT2* in cancer development and cellular metabolism is currently under investigation. The catalytic activity of *NAT2* significantly affects the cellular concentration of the cofactor acetyl-coenzyme A, which in turn may influence various cellular processes. Coenzyme A is a universal coenzyme involved in both anabolic and catabolic processes, and is involved in the metabolism of numerous compounds, including proteins, lipids, and carbohydrates. Recent studies have also suggested that acetylation may play a role as an epigenetic regulatory mechanism [17].

Of particular interest is the role of *NAT2* in connective tissue metabolism. A study published in 2016 by Kresyun V.J. et al. investigated the risk of abdominal adhesions based on *NAT2* polymorphisms. The study demonstrated the prognostic value of *NAT2* genetic polymorphisms in determining the risk of postoperative abdominal adhesions in children with connective tissue dysplasia phenotypes. Based on the results,

fast acetylators were identified as a high-risk group for developing adhesive intestinal obstruction and, therefore, it was recommended to implement comprehensive preventive measures starting from the intra-operative stage [18].

Intervertebral disc degeneration, a major cause of lower back pain, is caused by disrupted extracellular matrix biosynthesis and turnover in the disc. Neurotropin™, a commonly used analgesic drug, has an additional anabolic effect on glycosaminoglycan synthesis in the nucleus pulposus cells. Previous studies have shown a correlation between the effect of Neurotropin™ on aggrecan levels and *NAT2* activity [19]. A more in-depth review of the role of *NAT2* in connective tissue metabolism can be found in our previous study [20].

Slow *NAT2* acetylation status is generally associated with an increased risk of adverse pharmacotherapy effects, especially hepatotoxicity [14]. Conversely, fast acetylation may lead to treatment failure due to rapid drug clearance and subsequent relapse of the disease [14]. Given the importance, as well as the scientific and practical relevance, of assessing the acetylation phenotype and elucidating its role in the metabolic processes of various tissues, particular attention is paid to studying the impact of *NAT2* polymorphisms on the development and progression of degenerative-dystrophic spinal conditions. However, only a few studies have been undertaken in this area, and much remains to be elucidated.

The rate of acetylation reactions can be predicted using molecular genetic methods by identifying the set of most common SNPs in the structural region of the *NAT2* gene [21]. These SNPs may reflect the variability of clinical variants of pathology, providing valuable information for predicting connective tissue responses to surgery, determining strategies for comprehensive fibrosis prevention, and optimizing therapy based on metabolic status. However, molecular genetic methods are time-consuming and require a PCR laboratory equipped with specialized equipment and trained personnel, as well as sufficient funds for expensive reagents and supplies.

Determination of the N-acetylation rate has often been used as a phenotypic marker to prevent the development of pharmacotherapy complications in slow acetylators, and conversely, to increase the dose for fast acetylators to achieve an effective drug concentration, given that the drug is more rapidly eliminated from the body [22].

Various methods have been proposed, including the use of isoniazid and sulfadimethoxine as well as body weight-adjusted loads of food and drinks (such as coffee and tea). According to the methodology proposed by Bulovskaya L.N. of the Research Institute for Children's Infections (St. Petersburg), the difference in uric acid clearance before and after the loading procedure was determined. A borderline value of 4.48 mL/min was taken as the minimum threshold for fast acetylation [23].

The most accurate method for determining the *NAT2* genotype is through the analysis of caffeine metabolite levels in urine using high-performance liquid chromatography or mass spectrometry. However, these methods are expensive, time-consuming, and require highly trained personnel [24].

AIM

To develop a cost-effective and straightforward method for assessing the acetylation phenotype using spectrophotometric measurements of the metabolic rate of caffeine, as a model drug.

MATERIALS AND METHODS

A single-center cohort study was conducted based on data from 52 patients aged 27 to 66 years (mean age 51.04 ± 9.671 years) with spinal canal and dural sac stenosis. Patients over 18 years old with spinal canal stenosis who were admitted to the Neurosurgery Department of the Irkutsk Scientific Center for Surgery and Traumatology for surgical treatment between 2021 and 2023 were eligible for the study. They were selected using the consecutive sampling method. The selection process is illustrated in Figure 1. The inclusion criteria included MRI and MSCT-verified indicators of ligamentum flavum thickening of ≥ 3.5 mm and spinal canal narrowing of less than 10 mm in diameter. Surgical treatment included decompression and reconstruction of the spinal canal using a transpedicular fixation system. Patients with infectious purulent diseases (M46.2, M46.3, M46.5, M46.9 ICD-10), patients with diabetes mellitus type 1 and 2 (E10 and E11 ICD-10), systemic lupus erythematosus (M32 ICD-10), rheumatoid arthritis (M05-M06 ICD-10), psoriasis (L40 ICD-10), gout (M10 ICD-10), and bronchial asthma (J45 ICD-10) were excluded from the study if they were taking glucocorticoid, allopurinol, or inhaled corticosteroid medication during the study period. The technology for determining acetylation phenotype was developed using data from this group of patients.

For independent validation of the test, data from 32 conditionally healthy individuals, residents of Irkutsk without any health complaints and with no obvious musculoskeletal disorders, comparable in age to the “experimental” group (mean age 51.52 ± 0.933 years), were used.

The inclusion criteria for the control group included the absence of any health complaints for the previous 3 months and the absence of obvious musculoskeletal pathologies (self-reported). The exclusion criteria were identical to those for the group of patients described above with stenosis.

Caffeine was selected as the test substance due to its relative safety compared to other substances used as “probes” for *in vivo* studies [25]. All patients

participating in the study provided voluntary, informed consent and received a detailed explanation of the procedures to ensure high-quality collection of biological samples. To standardize caffeine consumption, subjects followed a caffeine-free diet for one day prior to and during the study, which included abstaining from tea, coffee, chocolate, bananas, cocoa, and other caffeinated beverages and products. After completing the caffeine-free period, blood and urine samples were collected. Blood samples were collected under sterile conditions in accordance with standard procedures from the cubital vein, into tubes containing clot activators, while avoiding vigorous shaking of the tubes or foaming of the contents.

Urine was collected between 6:00 p.m. and 10:00 a.m. the next day and stored at $6-8^{\circ}\text{C}$ until delivery to the laboratory. The total volume of urine collected during this period was measured using certified, calibrated graduated cylinders with an accuracy of 1 mL. The next day, after a physician's consultation, blood pressure was measured and the caffeine dose was calculated at 4 mg/kg body weight. Following the administration of caffeine as a test substance at 6 p.m., biological samples were collected: blood (in the morning) and urine (between the time of the single caffeine dose and 10 a.m. the next day). All samples were analyzed according to the following protocol: creatinine and uric acid levels were measured in blood serum and urine optical density (OD) at 272 nm, as well as creatinine and urate levels in urine after calculating its exact volume. Calculations were performed based on the data that was obtained.

Urine spectrophotometry. A sample of urine was subjected to centrifugation for 15 minutes at 1,500 rpm, and the resulting clear supernatant was collected. The optical density of the supernatant at 272 nm, corresponding to the absorption maximum of caffeine and its metabolites, was then measured using an SF-2000 spectrophotometer (Russia).

Biochemical analysis of blood and urine. In order to conduct the study, blood and urine samples were collected (1 – before caffeine intake, 2 – after caffeine administration). Creatinine and uric acid levels were measured using a Sapphire 400 automated biochemical analyzer (Japan). For urine analysis, samples were diluted 10-fold for uric acid determination and 100-fold for creatinine measurement to ensure accurate readings within the specified range. Commercial kits from Vital (St. Petersburg, Russia) were utilized for the quantitative analysis of creatinine and uric acid. The quality control of the study was performed in accordance with the current guidelines of the Ministry of Health of the Russian Federation.

The coefficient of variation (CV), used to determine the accuracy of biochemical parameter measurements, was calculated using the following formula $CV = (\sigma / \mu) \times 100 \%$, where σ is the sample standard deviation and μ is the sample arithmetic mean. The CV for each measured parameter did not exceed 5.7 %.

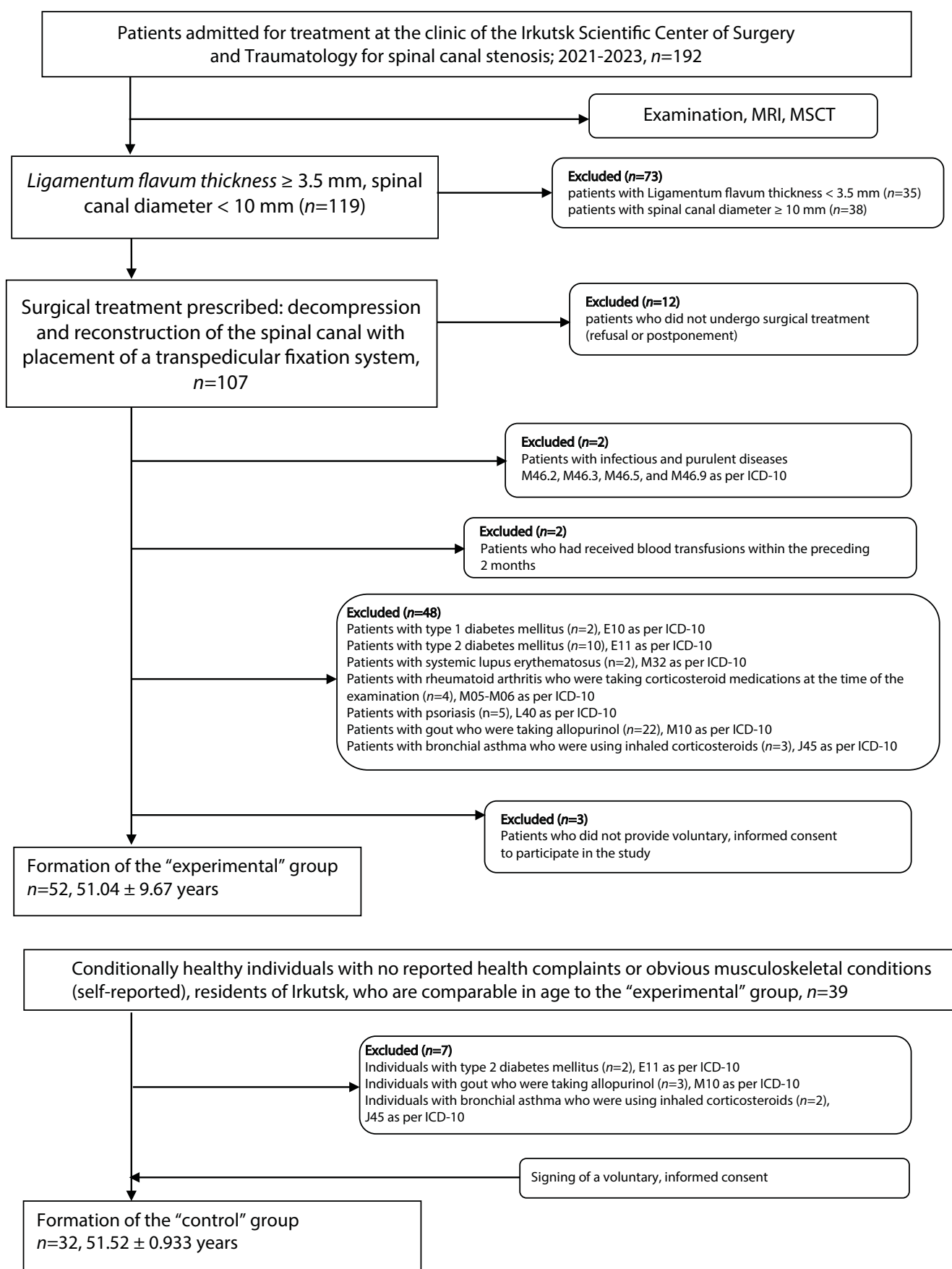


FIG. 1.
Block scheme of study participant selection

To verify the accuracy of the biochemical analyte measurements, two levels of commercially available control sera were used, in accordance with relevant regulatory guidelines for clinical diagnostic laboratories.

Calculation of the integral predictive indicator. The acetylation phenotype was determined based on the calculation of an integral predictive index using data on uric acid levels before and after administration of caffeine as a test substance, with adjustment for functional renal status based on creatinine clearance measurements. Additionally, spectrophotometric measurements at $\lambda = 272$ nm were taken into account.

Clearance of uric acid and creatinine was calculated using the formula:

Clearance of substance =

$$\frac{\text{concentration of substance in urine}}{\text{concentration of substance in blood}} \times \text{volume of urine per minute}$$

To adjust the contribution of each indicator in the prediction based on its significance for the study, we have proposed an integral predictive indicator (IPI), which is calculated using the following formula:

$$IPI = \frac{\left[\frac{Cl - UA}{Cl - Kr} \times OD_{272} \times 100 + a + b \right]}{BSA}$$

where *Cl-UA* is uric acid clearance after caffeine intake; *Cl-Kr* is creatinine clearance after caffeine intake; *a* is an additional score equal to 1 if the uric acid concentration has increased compared to the baseline, otherwise *a* = 0; *b* is an additional score equal to 1 if the urine optical density at $\lambda = 272$ nm has increased compared to the baseline, otherwise *b* = 0. *BSA* is the body surface area, calculated using the Mosteller formula:

$$BSA = \sqrt{\frac{\text{height, cm} \times \text{weight, kg}}{3600}}$$

The accuracy of the results obtained may be influenced by the temperature during transportation of biological material to the laboratory, therefore, strict adherence to temperature regulations is essential. It is also crucial to collect the entire volume of urine excreted over the specified time period, as the loss of even a small amount of urine can significantly impact the results and introduce errors.

Determination of NAT2 gene polymorphisms (reference method for acetylation rate assessment). To investigate single nucleotide polymorphisms in the structural region of the *NAT2* gene, a real-time polymerase chain reaction (RT-PCR) technique was employed. Blood samples were collected from the cubital veins. Genomic DNA was extracted from blood nucleated cells using the Nomotek-Extract DNA-2 reagent kit (Nomotek LLC, Ref R1003, Russia) following lysis and DNA purification on microcentrifuge columns.

A negative extraction control was utilized during each DNA extraction to monitor for potential contamination. The obtained genomic DNA preparation was then used to detect *NAT2* SNPs by performing specific RT-PCR reactions using the GenTest-M *NAT2* reagent kit (Nomotek LLC, cat. R2006, Russia). The following most common *NAT2* polymorphisms were identified: C282T (rs1041983), T341C (rs1801280), C481T (rs1799929), G590A (rs1799930), A803G (rs1208), and G857A (rs1799931).

Negative extraction controls were employed in each assay, along with positive control samples ("wild-type" homozygotes, heterozygotes, mutant homozygotes) containing a mixture of synthetic DNA templates containing the genotype sequences of the target polymorphisms, in order to monitor PCR amplification and ensure accurate genotype determination.

The isolated DNA was stored between -28°C to -20°C until PCR was performed. Specific PCR was also conducted on negative control samples obtained during the DNA extraction process, which had undergone all stages of the protocol, in order to identify any potential extraction issues.

Conditions for amplification were determined in accordance with the manufacturer's instructions for the test system. Fluorescence measurements were made using the FAM channel.

A threshold of 50 was set on the CFX96 real-time PCR system (Bio-Rad, USA). The analysis was deemed valid if the DNA concentration in the test sample exceeded 1 ng/ μL ($C_q \leq 30$), and all parameters specified by the reagent manufacturer were met.

Analytical characteristics of the method were assessed in both study groups. The sensitivity (Se) of the test was calculated using the formula: $Se = TP / (TP + FN) \times 100$, where TP is True Positives (fast acetylators correctly diagnosed) and FN is False Negatives (fast acetylators incorrectly classified as slow). The specificity (Sp) of the method was calculated using the formula: $Sp = TN / (TN + FP) \times 100$, where TN is True Negatives (slow acetylators correctly classified using the developed method) and FP is False Positives (slow acetylators erroneously classified as fast).

Ethical approval. The study protocol was approved by the Committee on Biomedical Ethics of the Irkutsk Scientific Center of Surgery and Traumatology (Protocol No. 9 dated December 16, 2021). All participants provided informed consent prior to their involvement in the study, ensuring voluntary participation.

Statistical analysis was performed using MS Excel 2022, Statistica version 10, and MedCalc version 23.4.0 for calculations, graphing, and ROC curve analysis. Differences between groups were considered significant at $p < 0.05$.

Cohen's *d* formula was used to calculate the effect size of the study:

$d = (M1 - M2) / S_p$, where *M1* and *M2* are the mean values of the fast and slow acetylator groups, respectively, and *S_p* is the pooled standard deviation. The pooled standard deviation was calculated using the formula:

$$\sigma_p = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}}$$

The pooled standard deviation for the patient group was

$$\begin{aligned} \sqrt{\frac{(30-1) \times 3,44^2 + (22-1) \times 1,94^2}{30+22-2}} &= \sqrt{\frac{29 \times 11,8336 + 21 \times 3,7636}{50}} \approx \\ &= \sqrt{\frac{343,1744 + 79,0356}{50}} \approx \sqrt{8,4442} \approx 2,906. \end{aligned}$$

The effect size (d) was also calculated using MedCalc software:

$$d = \frac{8,19 - 4,95}{2,906} \approx \frac{3,24}{2,906} \approx 1,115$$

Using the given data, knowledge of the effect size, number of subjects, and selection of a significance level of $\alpha = 0.05$, statistical power was calculated using the MedCalc software.

The optimal cut-off value on the ROC curve was determined by calculating the Youden index for each suitable point in the upper left corner of the ROC curve using MedCalc according to the formula $J = Se + Sp - 1$. The point with the highest Youden index was selected, and its coordinates were identified.

RESULTS AND DISCUSSION

Characteristics of the study groups are presented in Table 1. Initially, among the 192 patients with spinal canal stenosis admitted during the study period, 52 individuals were selected based on the criteria described (Materials and Methods). Of the 39 initially selected control group participants, only 7 were excluded after initial interview and questionnaire due to concomitant conditions or medications that could affect the biochemical analysis. The two groups were similar in terms of key characteristics. All participants underwent a comprehensive examination and none withdrew from the study at any stage. The study was conducted in its entirety, with no missing data.

The results of the reproducibility assessment for uric acid and creatinine determinations, obtained by analyzing the control material ten times within and between analytical runs, are presented in Table 2.

The coefficient of variation (CV) for uric acid ranged from 0.84–0.71 % to 5.2–7.3 %, and for creatinine it was between 3.8–4.2 % and 4.1–5.2 %. Within-run and between-run CV values indicate an acceptable level

of analytical reliability for the determination of these biochemical parameters.

Tables 3–6 present the values obtained for the experimental and control groups before and after caffeine intake. Significant differences between fast and slow acetylators were found in both groups only for urine optical density at 272 nm and the integrated IPI. To develop an integral predictive indicator, we aimed to combine the changes in uric acid clearance, adjusted for creatinine clearance and urine optical density at 272 nm, to provide a convenient method for calculating the rate of acetylation reactions. Together, these indicators can provide a more accurate prediction of the rate of metabolic reactions in individuals.

With an effect size of $d \approx 1.115$, and a number of participants $n_1 = 30$ (fast acetylators) and $n_2 = 22$ (slow acetylators), the statistical power was 0.95. That is, the probability of detecting an effect (difference in IPI between groups) was close to 95%.

The ROC curve (Fig. 2) illustrates the relationship between correctly classified positive cases (fast acetylators) and incorrectly classified negative cases (slow acetylators) among patients with spinal canal stenosis, with PCR analysis for *NAT2* polymorphisms serving as the reference standard. The area under the ROC curve (AUC) was 0.870 ($p < 0.001$).

When selecting the method for calculating the IPI, we compared various calculation methods (with and without consideration of body surface area) (Fig. 3).

The ROC curve constructed for the IPI, calculated with consideration of body surface area, extended slightly further into the upper left corner, which was confirmed by a larger AUC. The AUC values for the two calculation methods (with and without consideration of body surface area) were 0.870 and 0.883, respectively. Since the AUC for the IPI calculation with consideration of body surface area was larger, we chose this calculation method as the most optimal.

Tables 7 and 8 present the actual acetylation type status of the study participants, with an assessment of the classification accuracy using the proposed method.

In the patient group with spinal canal stenosis, the sensitivity of the developed method was $Se = 93.3\%$, and the specificity was $Sp = 90.91\%$.

In the control group, two individuals with the fast acetylation phenotype were incorrectly classified as having the slow acetylation phenotype using the proposed methodology, and one individual with the slow acetylation phenotype was classified as having fast acetylation (Table 6). These errors may be attributed to unaccounted for errors in the preanalytical phase, as well as differences between genotype and phenotype, or insufficient optimization of the method's sensitivity.

The lowest level of sensitivity was observed in the control group ($Se = 89.47\%$), which may be due to its smaller sample size. However, in both groups, the sensitivity and specificity values were both within acceptable ranges.

TABLE 1
CHARACTERISTICS OF THE PATIENT GROUPS FORMED FOR THE STUDY

Parameter	Patients with spinal canal stenosis, <i>n</i> = 52	Conditionally healthy individuals, residents of Irkutsk (control), <i>n</i> = 32
Age, years	51.04±9.671	53.32±0.933
Men, <i>n</i> (%)	29 (55.8)	14 (43.8)
Women, <i>n</i> (%)	23 (44.2)	18 (56.3)
Caucasians, <i>n</i> (%)	52 (100)	32 (100)
Fast acetylators, <i>n</i> (%)	30 (57.7)	19 (59.4)
Slow acetylators, <i>n</i> (%)	22 (42.3)	13 (40.6)
Hypertension stage 0, <i>n</i> (%)	24 (46.15)	16 (50.0)
Comorbidities (outside of exacerbation)		
Hypertension stage 1, <i>n</i> (%)	5 (9.62)	5 (15.6)
Hypertension stage 2, <i>n</i> (%)	19 (33.54)	6 (18.8)
Hypertension stage 3, <i>n</i> (%)	4 (7.69)	3 (9.4)
Cholelithiasis (calculous cholecystitis), <i>n</i> (%)	4 (7.69)	2 (6.3)
Bronchial asthma (remission), <i>n</i> (%)	1 (1.92)	0 (0)
Rheumatoid arthritis (remission), <i>n</i> (%)	1 (1.92)	0 (0)
Gout (remission), <i>n</i> (%)	1 (1.92)	0 (0)
History of allergies, <i>n</i> (%)	1 (1.92)	2 (6.3)

Notes. *Age data are presented as mean ± standard error of the mean.
Limitations: In the control group, data on comorbidities were self-reported.

TABLE 2
INDICATORS OF ANALYTICAL RELIABILITY OF URIC ACID AND CREATININE DETERMINATION

Analyte	Analytical reliability indicator	Within-run			Between-run		
Uric acid	Mean	211.3	215.4	214.8	218.4	219.9	210.4
	SD	6.8	4.9	7.2	7.3	6.8	5.2
	CV (%)	0.84	0.52	0.71	0.86	1.1	1.14
Creatinine	Mean	98.3	102.8	99.7	102.3	99.5	108.3
	SD	3.2	2.8	4.1	4.3	5.1	5.7
	CV (%)	3.8	2.3	4.2	4.1	4.8	5.2

Notes. SD – standard deviation; CV – coefficient of variation.

The acetylation status was confirmed using a reference method for determining the six most common polymorphisms [rs1041983, rs1801280, rs1799929, rs1799930, rs1208, rs1799931]. Various combinations of these selected polymorphisms enabled the identification of both slow and fast alleles. The concordance between genotype and phenotype varied between 89.47 % and 93.3 % (Tables 6 and 7), which demonstrates the effectiveness of this method. As a result, we achieved

sufficient sensitivity and specificity in determining the acetylation phenotype using this technology.

As a cut-off value for the optimal separation of fast and slow acetylators using the Youden index in MedCalc, a value of 6.8 was calculated. This cut-off point was chosen as it is located closest to the upper left corner of the ROC curve and is maximally distant from the diagonal, which is considered the best compromise between sensitivity and specificity for binary classification.

When assessing the calculated IPI values, all patients with an IPI below 6.8 were classified as slow acetylators, and patients with an IPI above 6.8 were classified as fast acetylators. Therefore, if the IPI is ≥ 6.8 , the acetylation phenotype is considered fast; if < 6.8 , it is considered slow.

Determination of the acetylation phenotype using the method described herein, employing caffeine as a test substance, allows for the prediction of acetylation reaction rates through blood and urine analysis. These rates are significant for connective tissue metabolism, including structures of the spinal motion segment and key adaptive mechanisms. The prediction of acetylation rates using a spectrometric approach for determining the acetylation phenotype based on caffeine metabolism is both effective and easy to perform. This method also provides valuable information for scientific research. By employing the proposed method, features of acetylation reactions can be identified more rapidly and inexpensively through analysis of blood and urine samples.

We have previously observed that, after caffeine intake, the clearance of uric acid and the OD of urine at 272 nm slightly increased in most fast acetylators. However, these changes were difficult to detect, as the measurement error in routine laboratory practice may have cancelled out these shifts. Therefore, we decided to consider all changing indicators in combination in order to achieve significant levels and assess the relationships with acetylation activity, in order to select the most appropriate ones. The determination of phenotype by uric acid clearance after caffeine intake has been used by other researchers [23, 26]; this method works well for patients without renal pathology. Chen X. et al. standardized the measurement of 14 caffeine metabolites by urinary creatinine, but used a different method [24]. Our proposed technology provides a more accurate determination, considering the functional excretory activity of the kidneys (by incorporating creatinine clearance into the calculation). Spectrophotometric measurement of the optical density at 272 nm corresponds to the absorption maximum absorption of caffeine and its major metabolites.

To date, the most accurate method for determining the NAT2 genotype remains the analysis of caffeine metabolites in urine using high-performance liquid chromatography or mass spectrometry [24]. However, these methods are quite expensive, labor-intensive, and require specially trained personnel.

There are also some inconsistencies with the theoretically expected results and limitations that need to be addressed. Caffeine stimulates diuresis by increasing renal blood flow and glomerular filtration rate. Therefore, it was expected that minute diuresis would increase after caffeine administration in our study [27–29]. However, no significant increase in minute diuresis was observed in any group; even if there were changes in some individuals, the significance levels were

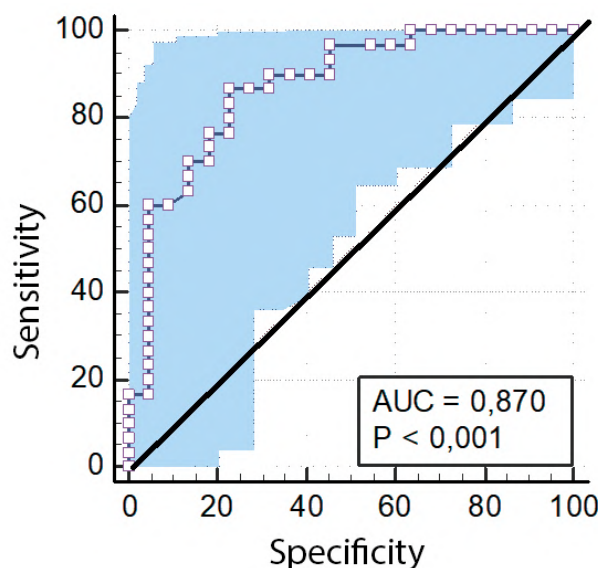


FIG. 2. ROC curve constructed based on the classification data for "fast" and "slow" acetylators of the "experimental" group using the proposed method with calculation of the integral predictive indicator and using real-time PCR as a reference method

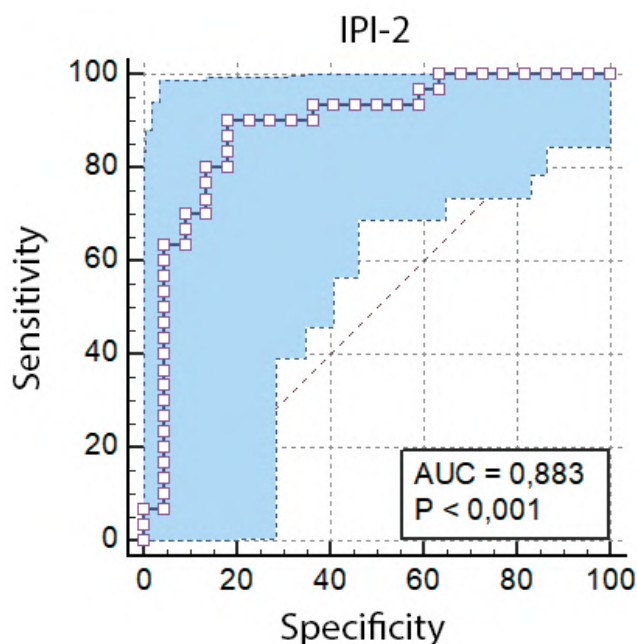


FIG. 3. ROC curve constructed based on the classification data for "fast" and "slow" acetylators of the "experimental" group using the calculation of the integral predictive indicator taking into account the body surface area (reference method – real-time PCR)

not achieved in the overall sample. This may be due to the small sample size, which is also a limitation of the study.

Caffeine is initially metabolized by cytochrome P450 (CYP1A2) to paraxanthine. Paraxanthine can then be further metabolized by NAT2 to acetylated products.

TABLE 3
INDICATORS OF THE "EXPERIMENTAL" GROUP BEFORE USING CAFFEINE AS A TEST DRUG, $n = 52$

Before caffeine intake	MD	Urinary UA	Serum UA	Urinary creatinine	Serum creatinine	OD-272	UA clearance	Creatinine clearance	Daily UA excretion	Daily creatinine excretion	Body surface area (BSA)
Reference values	0.5–1.5 mL/min	1.48–4.43 mmol/day	Male: 210–420, Female: 150–350 μ mol/L	Male: 7.1–17, Female: 5.6–15.8 mmol/L	Male: 72–115, Female: 58–96 μ mol/L	-	5.4–9 mL/min	64–126 mL/min	1.48–4.43 mmol/day	8.84–13.3 mmol/day	Mosteller formula: $\sqrt{[(\text{height, cm} \times \text{weight, kg}) / 3600]}$
Mean	1.067	2223.18	304.73	8154.95	88.74	0.1322	7.947	96.91	3.41	12.31	1.988
SD	0.151	376.67	50.21	1159.11	8.62	0.0346	1.956	9.96	0.74	0.73	0.213
SEM	0.028	68.77	9.17	211.62	1.57	0.0063	0.357	1.82	0.14	0.13	0.039
Mean	1.01	2116.89	286.49	8954.81	86.16	0.08	7.54	103.69	3.07	12.73	1.985
SD	0.19	416.68	35.39	1433.73	10.95	0.03	2.10	10.78	0.81	0.79	0.215
SEM	0.04	88.84	7.55	305.67	2.34	0.01	0.45	2.30	0.17	0.17	0.046
Differences between "fast" and "slow" acetylators, $p < 0.05$	-	-	-	-	-	$p < 0.05$	-	-	-	-	-

TABLE 4
INDICATORS OF THE EXPERIMENTAL GROUP AFTER CONSUMING CAFFEINE AS A TEST DRUG, $n = 52$

Before caffeine intake	MD	Urinary UA	Serum UA	Urinary creatinine	Serum creatinine	OD-272	UA clearance	Creatinine clearance	Daily UA excretion	Daily creatinine excretion	Body surface area (BSA)
Reference values	0.5–1.5 mL/min	1.48–4.43 mmol/day	Male: 210–420, Female: 150–350 μ mol/L	Male: 7.1–17, Female: 5.6–15.8 mmol/L	Male: 72–115, Female: 58–96 μ mol/L	-	5.4–9 mL/min	64–126 mL/min	1.48–4.43 mmol/day	8.84–13.3 mmol/day	Mosteller formula: $\sqrt{[(\text{height, cm} \times \text{weight, kg}) / 3600]}$
"Fast" acetylators (identified by PCR), $n = 30$											
Mean	1.115	2269.253	312.990	8201.443	86.587	0.190	8.181	104.584	3.615	12.954	8.19
SD	0.163	355.424	47.420	1117.335	8.207	0.142	1.765	10.835	0.627	1.044	3.44
SEM	0.030	64.891	8.658	203.996	1.498	0.026	0.322	1.978	0.115	0.191	0.63
"Slow" acetylators (identified by PCR), $n = 22$											
Mean	1.045	2187.800	300.145	8975.868	86.041	0.107	7.661	107.284	3.273	13.161	4.95
SD	0.194	422.009	36.792	1434.741	10.668	0.037	2.038	11.008	0.839	0.867	1.94
SEM	0.041	89.973	7.844	305.888	2.274	0.008	0.434	2.347	0.179	0.185	0.41
Differences between "fast" and "slow" acetylators, $p < 0.05$	-	-	-	-	-	$p < 0.05$	-	-	-	-	$p < 0.05$

TABLE 5
INDICATORS OF THE CONTROL GROUP BEFORE USING CAFFEINE AS A TEST DRUG, $n = 32$

Before caffeine intake	MD	Urinary UA	Serum UA	Urinary creatinine	Serum creatinine	OD-272	UA clearance	Creatinine clearance	Daily UA excretion	Daily creatinine excretion	Body surface area (BSA)
Reference values	0.5–1.5 mL/min	1.48–4.43 mmol/day	Male: 210–420, Female: 150–350 μ mol/L	Male: 7.1–17, Female: 5.6–15.8 mmol/L	Male: 72–115, Female: 58–96 μ mol/L	-	5.4–9 mL/min	64–126 mL/min	1.48–4.43 mmol/day	8.84–13.3 mmol/day	Mosteller formula: $\sqrt{[(\text{height, cm} \times \text{weight, kg}) / 3600]}$
Mean	1.054	2238.03	294.60	8414.84	88.57	0.1309	8.152	99.24	3.389	12.54	1.998
SD	0.160	379.43	50.89	1070.61	9.43	0.0366	1.966	9.85	0.766	0.61	0.194
SEM	0.037	87.05	11.68	245.61	2.16	0.0084	0.451	2.26	0.176	0.14	0.045
Mean	1.02	2129.83	279.48	9006.59	83.29	0.0880	7.80	108.20	3.09	12.84	2.020
SD	0.20	389.37	35.75	1669.47	10.00	0.0330	1.93	11.07	0.65	0.55	0.198
SEM	0.06	107.99	9.92	463.03	2.77	0.0092	0.54	3.07	0.18	0.15	0.055
Differences between "fast" and "slow" acetylators, $p < 0.05$	-	-	-	-	-	$p < 0.05$	-	-	-	-	-

Notes. MD – minute diuresis; UA – uric acid; OD-272 – optical density of urine after preliminary centrifugation measured at 272 nm; BSA – body surface area; SD – standard deviation; SEM – standard error of the mean; data are presented as arithmetic mean $\pm$ standard error of the mean.

TABLE 6
INDICATORS OF THE CONTROL GROUP AFTER CONSUMING CAFFEINE AS A TEST DRUG, $n = 32$

Before caffeine intake	MD	Urinary UA	Serum UA	Urinary creatinine	Serum creatinine	OD-272	UA clearance	Creatinine clearance	Daily UA excretion	Daily creatinine excretion	Body surface area (BSA)
Reference values	0.5–1.5 mL/min	1.48–4.43 mmol/day	Male: 210–420, Female: 150–350 μ mol/L	Male: 7.1–17, Female: 5.6–15.8 mmol/L	Male: 72–115, Female: 58–96 μ mol/L	-	5.4–9 mL/min	64–126 mL/min	1.48–4.43 mmol/day	8.84–13.3 mmol/day	Mosteller formula: $\sqrt{[(\text{height, cm} \times \text{weight, kg}) / 3600]}$
"Fast" acetylators (identified by PCR), $n = 19$											
Mean	1.090	2280.2	304.03	8367.3	85.68	0.1990	8.265	105.49	3.549	12.90	8.22
SD	0.165	343.2	45.85	1047.9	9.13	0.1788	1.776	10.46	0.595	0.72	3.99
SEM	0.038	78.73	10.52	240.41	2.09	0.0410	0.407	2.40	0.137	0.17	0.917
"Slow" acetylators (identified by PCR), $n = 13$											
Mean	1.053	2210.54	293.10	9035.08	84.15	0.1050	7.925	110.84	3.297	13.26	4.84
SD	0.209	405.37	38.66	1665.38	11.16	0.0381	1.860	12.46	0.676	0.58	2.08
SEM	0.058	112.43	38.66	461.89	3.09	0.0106	0.516	3.46	0.188	0.16	0.578
Differences between "fast" and "slow" acetylators, $p < 0.05$	-	-	-	-	-	$p < 0.05$	-	-	-	-	$p < 0.05$

Notes. MD – minute diuresis, UA – uric acid, BSA – body surface area; data are presented as arithmetic mean $\pm$ standard error of the mean.

TABLE 7

THE ACTUAL CONDITION OF THE OBJECTS OF THE “TRAINING” SAMPLE (PATIENTS WITH SPINAL CANAL STENOSIS), $n = 52$

Classification	Positive (“Fast”)	Negative (“Slow”)
“Fast”	True Positive (TP) 28	False Positive (FP) 2
“Slow”	False Negative (FN) 2	True Negative (TN) 20
Sensitivity Se = 93.3%, specificity Sp = 90.91%		

TABLE 8

THE ACTUAL CONDITION OF THE OBJECTS IN THE TEST SAMPLE (CONTROL GROUP), $n = 32$

Classification	Positive (“Fast”)	Negative (“Slow”)
“Fast”	True Positive (TP) 17	False Positive (FP) 1
“Slow”	False Negative (FN) 2	True Negative (TN) 12
Sensitivity Se = 89.47%, specificity Sp = 92.3%		

Cytochrome P450 enzymes, like *CYP1A2*, can produce reactive oxygen species. On the other hand, peroxy-nitrites and superoxides produced by mitochondria can inhibit *NAT2* irreversibly [30].

The difficulty in unambiguously interpreting the results lies in the fact that the uricase enzyme used in the biochemical determination of uric acid (Vital test system, Russia) has a high but not absolute level of specificity. Therefore, although at a slower rate and with a lower affinity for the substrate, this enzyme could theoretically also oxidize other xanthines with similar structures, including caffeine metabolites. As a result, some of the H_2O_2 involved in the formation of a measurable color would come not only from uric acid, but also from caffeine metabolites. On the other hand, it is known that caffeine and its metabolites inhibit the activity of xanthine oxidase, an enzyme that acts in parallel with *NAT2*. Inhibition of xanthine oxidase may increase the need for active *NAT2* activity [31]. Thus, theoretically, two opposing effects may occur: (1) caffeine metabolites in a blood sample (*in vitro*) could cause falsely elevated uric acid results due to interaction with uricase in the reaction mixture; and (2) the physiological effect of caffeine (*in vivo*) on the other hand, could suppress uric acid production due to xanthine oxidase inhibition [31]. While studying the fine mechanisms of caffeine excretion was not the focus of our research, we attempted to address potential challenges associated

with preanalytical and analytical laboratory processes. To address possible difficulties, conflicting results, and inaccuracies in analysis, additional studies with larger sample sizes and validation of the developed method using an independent patient population are necessary.

CONCLUSION

Determination of the acetylation phenotype through the calculation of an individual predictive indicator based on data from uric acid and creatinine measurements, adjusted for urinary optical characteristics after caffeine administration, considering the dynamics of these indicators' changes, enables, through blood and urinary analysis, determination of the acetylation reaction rate to predict treatment outcomes based on *NAT2* polymorphisms, and identification of risk groups. This study allows the identification of individual variations in connective tissue metabolism, which can be used to search for new connections in pathogenesis and explore the mechanisms of pain and fibrosis. Nevertheless, the relatively small number of individuals studied with this method imposes certain limitations; validation in larger-scale studies is recommended. It should also be noted that strict adherence to the preanalytical phase of laboratory testing is crucial for obtaining reliable results.

The proposed algorithm, based on blood and urine testing, allows for the prediction of the rate of acetylation reactions, which are actively involved in connective tissue metabolism and may also play a role in pain and fibrogenic processes. This method aids in the identification and characterization of metabolic variations in the course of degenerative-dystrophic spine pathology, and it may also offer insights into preventing epidural fibrosis, as well as providing predictions for surgical outcomes based on the NAT2 genetic polymorphism.

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Conflicts of interest

The authors declare no conflicts of interest.

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