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Cytochrome P450 3A4 activity and genetic variants as predictors of liver failure in patients with obstructive jaundice

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ABSTRACT

Liver failure in patients with obstructive jaundice is a significant contributor to mortality within this patient cohort. The exact mechanism and triggers of this occurrence are yet to be fully understood. With this in mind, our study aimed to assess the correlation between the urinary 6 β-OHC/C ratio and various biochemical parameters of liver function. Furthermore, we conducted genotyping of CYP3A4*22 (rs35599367), CYP3A5*3 (rs776746) polymorphic markers to investigate the potential effects of their variants on the probability of liver failure in obstructive jaundice. Our study included 75 patients diagnosed with severe obstructive jaundice. All test subjects underwent functional liver tests, and control blood tests were administered on the seventh day following biliary decompression. Patients were categorized into two groups: group 1 - patients without liver failure (n = 60) and group 2 - patients with liver failure (n = 15). Laboratory indexes such as 6 β –OHC concentration and 6 β- OHC/cortisol ratio can serve as significant predictors of liver failure in patients with moderate and severe degree obstructive jaundice after biliary decompression. Based on the study of "wild" and polymorphic variants of CYP3A4*22 (CC and CT) and polymorphism of CYP3A5*3A6986G (GG, GA, AA), it was discovered that liver failure in the CYP3A4*22 variant may be associated with the CC genotype, and in the CYP3A5*3 variant - with the GA genotype. Hence, the determination of 6β- OHC concentration and 6β- OHC/C ratio, as well as the analysis of polymorphic and "wild" variants of CYP3A4*22 (CC and CT) and CYP3A5*3 polymorphism A6986G (GG, GA, AA), may play a crucial role in predicting liver failure in patients with obstructive jaundice.

1. Introduction

Cytochrome P450 (CYP450) enzymes are heme monooxygenases responsible for the metabolism of numerous xenobiotics. They activate metabolism, converting these compounds into water-soluble and less toxic substances that are easily eliminated from the body [1,2]. CYP3A is a subfamily of cytochrome P450 isoenzymes with the highest expression in humans, and it includes isoforms 3A4, 3A5, 3A7 and 3A43 [3]. The CYP3A subfamily plays an important role in the biotransformation of more than 50% of drugs used in clinical practice [4]. CYP3A4 is the most common isoform expressed in the liver and intestine with high individual variability in protein level and catalytic activity [5]. Several noninvasive methods have been proposed to assess CYP3A4 activity: the erythromycin breath test, the urinary dapsone recovery test, the measurement of midazolam clearance, and the measurement of the ratio of endogenous urinary 6 β-hydroxycortisol (6 β-OHC) to free cortisol (C) [6–9]. Recent studies have shown that using the urinary 6 β-hydroxycortisol to free cortisol ratio (6 β-OHC/C) is the most sensitive indicator of CYP3A induction [10–12]. There have been several mentions of experience with the 6 β-OHC/C ratio in liver cirrhosis [13]. However, the use of this method in patients with obstructive jaundice is lacking. Liver failure in patients with obstructive jaundice could be induced by the changes of liver enzymes and potentially by the activity of cytochrome P450. Activity of the cytochrome P450 under these conditions could be restricted by the overproduction of the metabolites and other factors. To test this, in the present study, we evaluated the 6 β-OHC/C ratio in urine samples from patients with obstructive jaundice. Sampling was done by collecting bodily fluids from patients admitted to the

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hospital with obstructive jaundice who met the inclusion/exclusion criteria. This ratio was used as a simple noninvasive biomarker to assess CYP3A4 activity. In addition, the correlation between the urinary 6 β-OHC/C ratio and various biochemical parameters of liver function was studied. In our study, we also performed genotyping of CYP3A4*22 (rs35599367), CYP3A5*3 (rs776746) polymorphic markers to study the possible effect of their variants on the probability of liver failure in obstructive jaundice.

2. Material and methods

2.1. Experimental design

The study included 75 patients with severe obstructive jaundice. Obstructive jaundice was diagnosed on the basis of clinical, laboratory and instrumental diagnostic tools. All subjects underwent functional liver tests, which included determination of total protein, bilirubin (total and direct), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea and creatinine. Coagulogram values were also investigated. The degree of severity of the obstructive jaundice was determined according to hyperbilirubinemia level. Patients received no known substances influencing CYP3A4 activity (for example, macrolide antibiotics, contraceptives or azole antifungal agents) before and during the study. Control blood tests were performed on day 7 after performing biliary decompression.

Inclusion criteria:

1. Men and women over 40 years old with obstructive jaundice.
2. Hyperbilirubinemia levels more than 100 μmol/l, but less than 500 μmol/l.
3. The absence of acute purulent cholangitis.
4. Adequate endoscopic biliary decompression with a plastic stent or adequate percutaneous biliary decompression with biliary drainage.
5. Signed informed consent to participate in the study.

Exclusion criteria:

1. Patients with cholangitis, biliary sepsis.
2. Patients who had any variant of biliary decompression previously performed which led to obstructive jaundice due to dysfunction of previously installed stent.
3. Patients with acute or chronic renal failure (both due to obstructive jaundice and renal disease).
4. Patients with multiple organ failure as a result of obstructive jaundice or cancer intoxication.
5. Patients with metastatic liver damage, ascites or disseminated cancer process.
6. Patients with decompensated general somatic pathology as a result of obstructive jaundice.
7. Patients with intolerance to iodine-containing contrast agents.
8. Patients with severe persistent coagulopathy, despite adequate blood component replacement therapy.

2.2. Collection of blood and urine samples

On the morning of the day of the study, urine samples were collected according to the generally accepted rules of urine collection between 08:00 and 12:00. The samples for each patient were then separated into portions and stored frozen at −80 °C without the addition of any preservatives until the time of analysis. For genotyping, the biological material for genomic DNA isolation was 4 μl of venous blood collected from the ulnar vein into a VACUETTE® vacuum tube (GreinerBio-One, Austria) containing EDTA-K2 or EDTA-K3. Samples were stored at −80 °C until DNA extraction.

2.3. Analysis of 6 β-hydroxycortisol (6 β-OHC) and free cortisol in urine

Concentrations of free cortisol and 6β-OHC in urine were determined using a combined high-performance liquid chromatography with mass spectrometric detection (HPLC/MS) method. An Agilent 6410 mass spectrometer, triple quadrupole type, was used. Ionization of molecules was performed in negative ionization mode on an electrospray. Temperature of drying gas (nitrogen) in the source was 350 °C, flow rate of drying gas was 11 l/min; pressure at the atomizer was 35 psi. The voltage at the capillary was 4000 V. Spectra were recorded in the mode of multiple molecular reactions registration. Chromato-mass spectrograms were recorded: for cortisol by product ion 331 *m/z* (precursor ion 407 *m/z*); for 6β- OHC by product ion 347 *m/z* (precursor ion 423 *m/z*). The voltage at the collisional cell electrodes was 15 V.

2.4. Instruments

We used an Agilent 1200 chromatograph, which includes the following units: a binary four-channel pump, an automatic sample introduction device (autosampler), and a thermostat for the chromatographic columns. Composition of the mobile phase: component “A" - 0.1% solution of formic acid in deionized water; component “B" - 0.1% solution of formic acid in acetonitrile. The flow rate was 0.4 ml/min. Chromatographic separation of the target compounds was performed in the gradient elution mode on an Agilent chromatographic column “Polaris-3 C18-A″, 3.0 mm × 50 mm, 3 μm grain size, with the following program: 0–1.5 min 10% “B"; 2 min 20% “B"; 6 min 60% “B"; 7 min 10% “B"; 7–10 min 10% “B". The thermostat temperature of the chromatography column was 40 °C. Under these conditions, the retention time of the analyzed compounds was 4.5 min (6β- OHC) and 7 min (cortisol).

2.5. Extraction method

Extraction of the target compounds from urine samples was performed using the liquid-liquid extraction method. A 5-fold excess of the mixture of ethyl acetate and isopropanol (85:15) was added to a 250 μl sample. Shaken for 15 min, the organic layer was separated and evaporated in a nitrogen current. The dry residue was reconstructed in the mobile phase, after which the chromatographic vials were placed in an autosampler for subsequent analysis. The injection volume was 5 μl. Quantification of the target compounds was performed using the absolute calibration method. Urine samples preliminarily purified from the endogenous content of the substances analyzed by the above method were used as a matrix for the preparation of standard solutions with known concentration. The quantification limit for cortisol and 6β- OHC was 1 ng/μl.

2.6. Genotyping of CYP3A4*22 (rs35599367), CYP3A5*3 (rs776746) polymorphic markers

Genotyping was performed at the Research Institute of Molecular and Personalized Medicine of the Russian Medical Academy of Continuing Professional Education. Biological material for genomic DNA extraction was 4 μl of venous blood collected from the ulnar vein into a VACUETTE® vacuum tube (GreinerBio-One, Austria) containing EDTA-K2 or EDTA-K3. Samples were stored at −80 °C until DNA extraction. Genomic DNA extraction from whole blood was performed using the S-Sorb reagent kit for DNA extraction on a silica sorbent (Syntol LLC, Russia). The concentration of extracted DNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, NY, USA). Carriage of the single nucleotide genetic polymorphism was determined by allele-specific real-time PCR on a CFX96 Touch Real Time System with CFX Manager software version 3.0 (BioRad, USA) using a commercial reagent kit for determining the CYP3A5*3 (G/A, rs776746) polymorphism (Syntol, LLC, Russia). For PCR, 20 μl of a mixture of components was used. According to the

manufacturer's instructions, 2.5 × Reaction mixture was added to each sample with 10 μl, 2.5 × Diluent with 10 μl, and Taq DNA polymerase, 5 E/μl, with 0.5 μl. 5 μl of DNA from the samples under study was added to each test tube. The amplification program consisted of an incubation step at 95 °C for 3 min, a denaturation step at 95 °C for 15 s, and an annealing step at 63 °C for 40 s for 39 cycles. The fluorescence signal developed through the appropriate channel: FAM and HEX. Carriage of the CYP3A4*22 (rs35599367) polymorphic marker was determined using a commercial kit TaqMan®SNP Genotyping Assays and TaqMan Universal Master Mix II, no UNG (Applied Biosystems, USA). According to the manufacturer's instructions, TaqMan®SNP Genotyping Assays 0.5 μl in a 40-fold dilution in 10 μl of TaqMan Universal Master Mix II, no UNG and 9.5 μl of RNase-free water were used. Five μl of DNA from the samples were added to each test tube. The amplification program included an incubation step at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s, and an annealing at 60 °C for 1 min for 49 cycles. The fluorescence signal developed through the appropriate channel: FAM and VIC.

2.7. Statistical analysis

The 6β-OHC/C ratio was expressed as the median value. Values and nonparametric tests such as the Mann-Whitney and *U* test were used for comparison. Regression analysis and analysis of variance were used to assess the possible correlation between urinary 6β-OHC/C ratio and any demographic factors (e.g., age, sex, and smoking) or biochemical and hemostaseology parameters (e.g., serum creatinine, bilirubin, INR, etc.). Statistical significance was considered at $p < 0.05$.

3. Results

The study encompassed a total of 75 patients. After conducting a comprehensive range of laboratory and instrumental tests, the clinical presentation of the ailment and degree of liver failure resolution were evaluated. Despite the implementation of adequate biliary decompression, 15 patients experienced liver failure, necessitating prolonged hepatotropic therapy and an extended hospitalization period. Accordingly, the patients were categorized into two distinct groups, namely group 1 - without liver failure and group 2 - with liver failure.

The laboratory test results were compiled into a comprehensive database, followed by a statistical analysis of the acquired data. Table 1 illustrates the characteristics of the patients included in the study.

Results of CYP3A4*22 (rs35599367), CYP3A5*3 (rs776746) polymorphic markers genotyping are shown in Fig. 1.

Results of laboratory tests of patients with obstructive jaundice with determination of CYP3A5*3A6986G (GG, GA, AA) polymorphism is shown in Fig. 2.

A comparative evaluation of the biochemical parameters in blood analysis and hemostaseology prior to decompression exhibited no noteworthy distinctions between the groups under comparison, implying that the compared groups were statistically homogeneous. The comparative outcomes of the aforementioned analysis have been documented in Table 2.

The next step was performing comparative analysis of 6β- OHC concentration, cortisol concentration and 6β- OHC/C ratio as predictors of liver failure in patients after biliary decompression. The results of the analysis are shown in Fig. 3.

A significant disparity was observed in the 6β-OHC concentration ($p = 0.048$) between Group 1 (patients without liver failure) and Group 2 (patients with liver failure). The average 6β-OHC concentration was higher in Group 1 (139.15 ng/ml) compared to Group 2 (114.27 ng/ml), and the median was also higher in Group 1 (125.78 ng/ml) than in Group 2 (64.64 ng/ml). Furthermore, the ratio of 6β-OHC concentration to cortisol concentration (6β-OHC/C ratio) also showed a considerable difference ($p = 0.0002$) between the two groups. This ratio was notably lower in patients with liver failure. The average ratio in Group 1 was 3.0159, while it was 1.4138 in Group 2. Similarly, the median ratio was 2.8247 in Group 1 and 1.5105 in Group 2. Based on these observed differences, we can conclude that the laboratory indices, specifically the 6β-OHC concentration and 6β-OHC/C ratio, could serve as robust predictors of liver failure in patients with moderate to severe grade obstructive jaundice following biliary decompression. These laboratory methods should be employed in clinical practice to forecast liver failure and promptly initiate appropriate hepatotropic therapy in large randomized trials.

On the 7th day following adequate biliary decompression, a comparative assessment of biochemical blood analysis and hemostaseology values was conducted and presented in Table 3.

When analyzing Table 3, statistically significant differences in the level of total ($p = 0,0082$) and direct bilirubin (0,0099) on the 7th day after biliary decompression were higher in the group of patients with hepatic failure. This can be explained from the position of its criteria definition. An increase of creatinine levels was noted in group 2. There were no statistically significant differences in other parameters in the studied groups.

Statistical analysis of results of genotyping of polymorphic and "wild" variants of CYP3A4*22 (CC and CT) and polymorphism of CYP3A5*3 (GG, GA, AA) was performed (Table 4; Figs. 4 and 5).

Based on the data in Table 4 and Figs. 4 and 5, hepatic failure in the CYP3A4*22 variant may be associated with the CC genotype and in CYP3A5*3 – with the GA genotype.

4. Discussion

We studied biochemical blood parameters on the 7th day after biliary decompression. Statistically significant difference in levels of total ($p = 0,0082$), direct bilirubin (0,0099) and prothrombin (0,0145) on the 7th day after biliary decompression was higher in the group of patients with liver failure. It was also noted that creatinine levels were higher in group 2. However, there were no significant differences in other parameters in the studied groups.

The 6b-OHC concentration and 6 β-OHC/C ratio were significantly lower in the group of patients with liver failure. This allows us to conclude that these laboratory parameters (6 β-OHC concentration and 6 β-OHC/C ratio) may be strong predictors of hepatic failure in patients with obstructive jaundice after biliary decompression. This fact is very important for further application in clinical practice, because doctors today have no possibility to predict the development of hepatic insufficiency in the early stages of obstructive jaundice. The pathogenesis of this process is directly related to the function of cytochrome R450. Waring R. et al. states that it directly affects the metabolism of steroid hormones, bile acids, polyunsaturated fatty acids [14]. Therefore, a change in cytochrome R450 activity is an important predictor of the development of liver failure. As a result, the use of hepatotropic drugs and other possible medications to prevent the development of liver failure begins in the late stages of the disease, which also significantly affects the results of treatment. In addition, the study of the effect of 6 β-OHC concentration and the 6 β- OHC/C ratio on the development of liver failure is also important for basic science, which can lead to understanding the pathogenesis of its development and outline targeted drugs for its correction. Thus, even before the development of liver failure,

Table 1
Characteristics of patients with obstructive jaundice included in the study.

	Group 1 (n = 60)	Group 2 (n = 15)	p	Total	Reference
Number	60	15		75	
Average age, years	69.7 ± 9.5	65.2 ± 14.7	0.478	68.2 ± 11.3	42–79
Sex (m/f)	32/28	9/6	0.128	41/34	
Smoking (yes/no)	17/43	4/11	0.218	21/54	

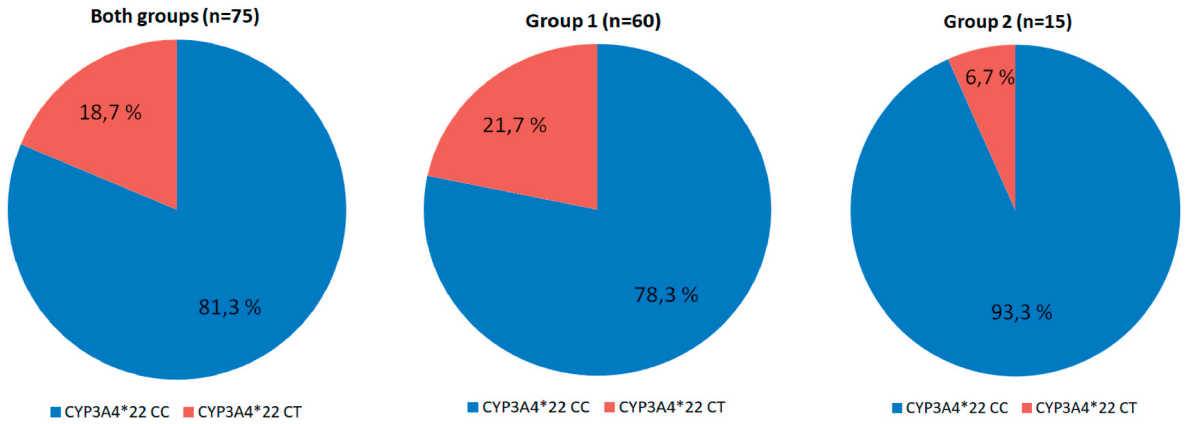

Fig. 1. Characterization of patients with obstructive jaundice with determination of "wild" and polymorphic variants of CYP3A4*22 (rs35599367) (CC and CT).

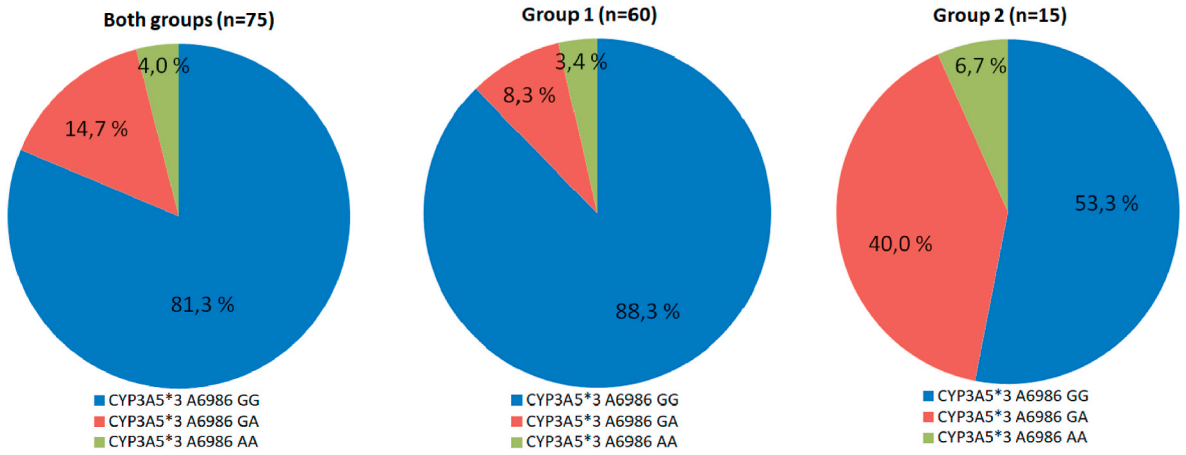

Fig. 2. Characterization of patients with obstructive jaundice with determination of CYP3A5*3 (rs776746) A > G polymorphism (GG, GA, AA).

Table 2
Comparative analysis of biochemical and hemostaseology blood parameters before biliary decompression.

Laboratory indicator	group 1 (n = 60)		group 2 (n = 15)		
	average	median	average	median	p
Total bilirubin level, μmol/L	329,99	311,50	306,7	309,2	0,9091
Direct bilirubin level, μmol/L	195,90	181,60	194,9	194,4	0,7317
AST level, U/L	177,67	148,50	96,8	86,0	0,1053
ALT level, U/L	180,32	124,80	110,8	85,7	0,2774
Urea level, mmol/l	5,82	5,40	5,4	6,0	0,9818
Creatinine level, μmol/L	92,93	85,50	96,8	95,0	0,3946
Prothrombin level, %	77,65	84,50	68,0	65,0	0,5984
Prothrombin time, s	18,06	13,30	18,3	15,4	0,6638
International normalized ratio	1,60	1,20	1,6	1,4	0,5059
Activated partial thromboplastin time, s	30,27	26,30	30,4	29,1	0,3447
Activated partial thromboplastin time (ratio)	0,98	0,87	0,9	0,9	0,6308
Fibrinogen, g/l	4,45	4,51	3,9	4,0	0,1285

based on an indirect study of the activity of the CYP3A4 isoform of cytochrome P450, it is possible to predict the development of liver failure with a high degree of probability. There is every reason to believe that the use of this method is promising for predicting liver failure in toxic and virus hepatitis. This allows us to recommend further larger-scale randomized studies in this topic for further use of these laboratory methods in clinical practice in order to predict the development of liver failure and the timely initiation of specific hepatotropic therapy to curb it.

Cytochrome P450 (CYP) enzymes metabolize approximately more than 70% of drugs for clinical use. Among them, CYP3A4 is quantitatively the most important P450 enzyme in adults. It is expressed to a major extent in the human liver (95%) thus contributing to pre-systemic and systemic metabolism of approximately 30% of all drugs. CYP3A4*22 appears to be the most clinically relevant common variant in CYP3A4 [15]. CYP3A4*22 is the main biomarker that predicts CYP3A4 activity [16]. However, the molecular genetic mechanisms underlying CYP3A4*22 effects remained to be elucidated. Previously, using CYP3A4 minigenes and rs35599367 as markers, showed that the minor rs35599367 T allele is associated with lower levels of heteronuclear RNA than the C allele in HepG2 cells [17]. On the basis of study of "wild" and polymorphic variants of CYP3A4*22 (CC and CT) it was found that liver failure in CYP3A4*22 variant could be connected with CC genotype. Thus, the "wild" variant of CYP3A4*22 (CC) can be considered as a predictor of liver failure in patients with obstructive jaundice.

CYP3A5 is one of the four CYP3A genes that encode the CYP3A subfamily of enzymes responsible for the metabolism of more than 50% of medicines prescribed worldwide [18]. The CYP3A5 expression level and enzymatic activity are modulated by genetic polymorphisms. The presence of single-nucleotide polymorphisms in intron 3 of CYP3A5 6986 A > G results in a loss of CYP3A5 activity because a splice site

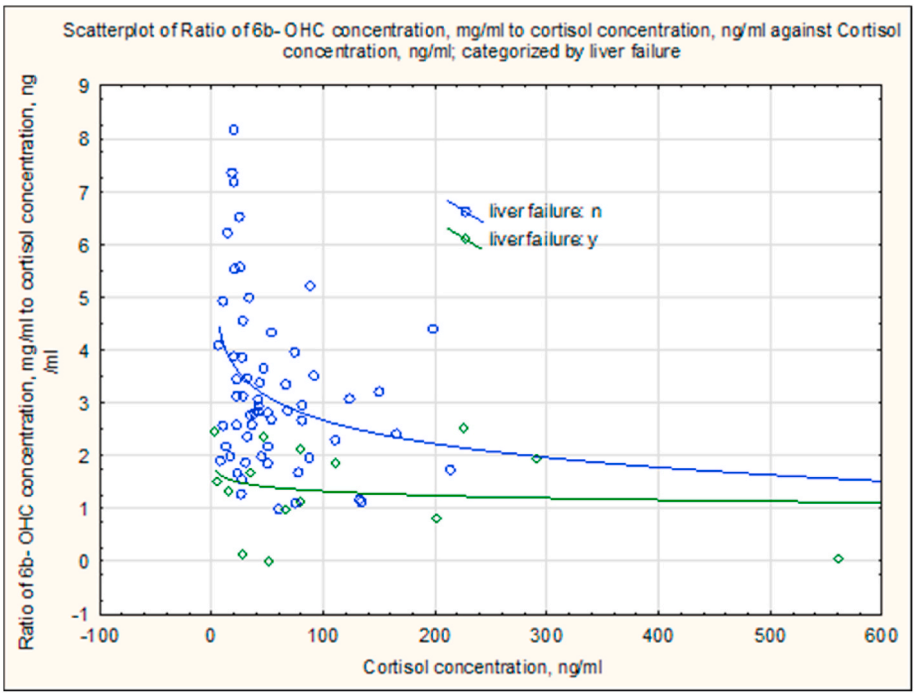

Fig. 3. Comparative analysis of 6β-OHC concentration, cortisol concentration and 6 β- OHC/C ratio before biliary decompression.

Table 3
Comparative analysis of biochemical and hemostaseology blood parameters on the 7th day after biliary decompression.

Laboratory indicator	group 1 (n = 60)		group 2 (n = 15)		
	average	median	average	median	p
Total bilirubin level, μmol/L	158,70	165,50	294,6	266,0	0,0082
Direct bilirubin level, μmol/L	87,33	89,00	173,1	136,0	0,0099
AST level, U/L	96,13	79,50	68,6	43,0	0,6975
ALT level, U/L	86,56	60,00	119,9	66,0	0,8730
Urea level, mmol/l	5,56	4,95	5,7	4,8	0,8016
Creatinine level, μmol/L	72,89	72,00	84,0	80,0	0,0855
Prothrombin level, %	78,02	80,80	53,3	59,0	0,0145
Prothrombin time, s	14,98	13,50	13,0	11,9	0,1851
International normalized ratio	1,32	1,18	1,6	1,5	0,4210
Activated partial thromboplastin time, s	29,25	28,85	35,5	34,2	0,5454
Activated partial thromboplastin time (ratio)	0,99	0,97	0,9	0,9	0,2988
Fibrinogen, g/l	4,11	4,16	4,1	4,0	0,8372

Table 4
Characteristics of patients with obstructive jaundice with determination of "wild" and polymorphic variants CYP3A4*22 (rs35599367) (CC and CT) and polymorphism CYP3A5*3 (GG, GA, AA).

	CYP3A4*22	CYP3A5*3	Group 1	Group 2 $_{Total}$	
	C > T	A > G	(n = 60)	(n = 15)	
Count	CC	GG	42	7	49
Row Percent			85,7%	14,3%	
Count	CC	GA	5	6	11
Row Percent			45,5%	54,5%	15,63%
Count	CC	AA	2	1	3
Row Percent			66,6%	33,3%	3,13%
Total Percent			*49*	*14*	*63*
Count			*77,8%*	*22,2%*	
Count	CT	GG	6	0	6
Row Percent			92,9%	7,1%	100,00%
Count	CT	GA	5	0	5
Row Percent			0,00%		0,00%
Count	CT	AA	2	1	3
Row Percent			0,00%		0,00%
Count			*11*	*1*	*12*
Column Percent			*10,00%*	*0,00%*	
Total Percent	**Total**		**60**	**15**	**75**
Count			**80,0%**	**20,0%**	

variant leads to a truncated inactive enzyme [19]. This genetic polymorphism may affect the metabolism of endogenous toxins in liver tissue. In our study we investigated that polymorphism of CYP3A5*3 A6986G (GG, GA, AA) by genotyping of venous blood it was found that liver failure in variant CYP3A5*3 could be associated with GA genotype. Thus, variant GA of CYP3A5*3 can be considered as a predictor of liver failure in patients with obstructive jaundice.

The identified patterns indicate the influence of genetic predisposing factors on the development of liver failure. In this case, it is impossible to influence the polymorphism of these sequences, but if they are identified, it may allow the physician to start intensive hepatotropic therapy without waiting for the development of liver failure, which may affect the probability of its development and prevent an adverse outcome of the disease.

5. Conclusion

Thus, the determination of 6β-OHC concentration and 6β-OHC/C ratio, coupled with the analysis of "wild" and polymorphic variants of CYP3A4*22 (CC and CT) and CYP3A5*3 polymorphism A6986G (GG, GA, AA), may prove to be a pivotal factor in the prediction of liver failure in patients with obstructive jaundice. These indices have the potential to serve as reliable clinical predictors of this complication, thereby enabling the timely initiation of specialized hepatotropic therapy during the early stages of treatment.

Statements and Declarations.

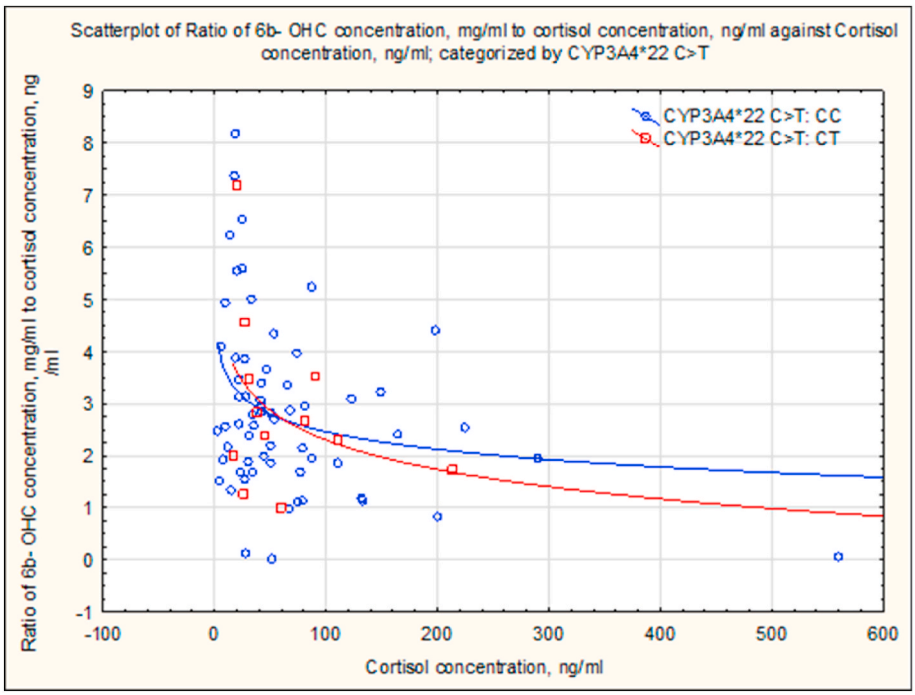

Fig. 4. Comparative analysis of 6β-OHC concentration, cortisol concentration and 6 β- OHC/C ratio for polymorphic and "wild" variants of CYP3A4*22 (CC and CT).

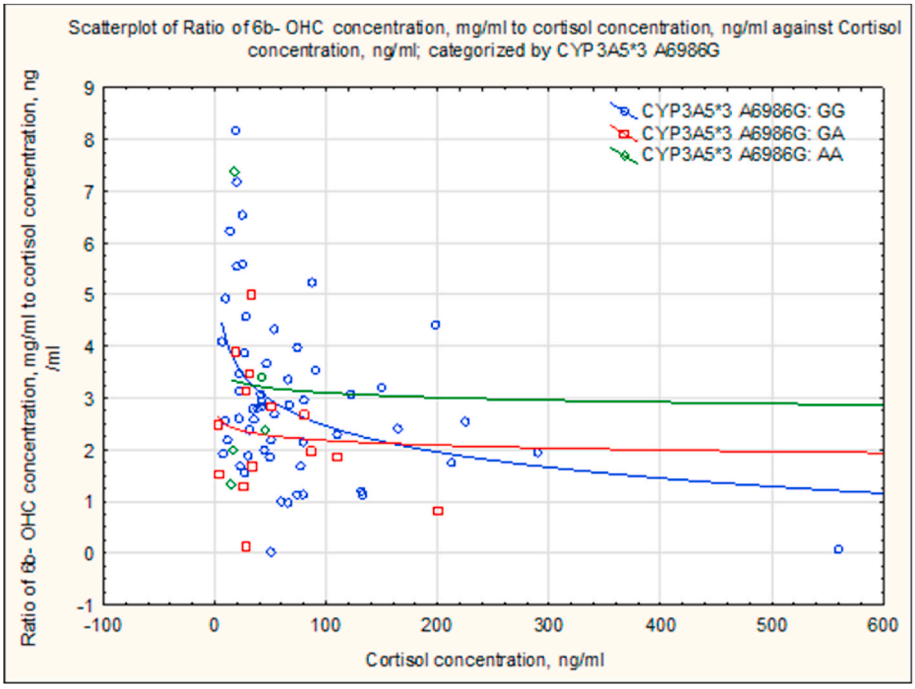

Fig. 5. Comparative analysis of 6β-OHC concentration, cortisol concentration and 6 β- OHC/C ratio for polymorphism of CYP3A5*3 (GG, GA, AA).

Author contributions

Dmitri Sychev, Alexey Shabunin: the concept of research, scientific guidance Sergey Lebedev: scientific management and the approval of the final version of the article, the writing of the text and editing. Mikhail Tavobilov, Alexey Karpov, Kirill Abramov, Pavel Bochkov, Roman Shevchenko, Natalia Denisenko: data collection, the writing of the text and editing. All authors took part in the discussion of the results and the formation of the final version of the article.

Ethics approval

Informed consent was obtained from all individual participants included in the study. The study was conducted in accordance with the 1964 Declaration of Helsinki and its later amendments, and the protocol was approved by the Ethics Committee of RMACPE of the Ministry of Health of the Russian Federation (No. 4751).

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Declaration of competing interest

The authors declare no conflict of interest. This article does not contain any studies with animals performed by any of the authors.

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