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Blood transcriptional signatures of gallstone pathogenesis in patients with cholelithiasis and choledocholithiasis

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Abstract

Aim: Disease-specific molecular signalling in peripheral blood has the potential to inform on pathophysiological mechanisms of diseases. Here, we aimed to investigate the blood-based gene expression profiles that reflect disease-specific pathogenic mechanisms of gallstones, such as antioxidant defence, heat-shock responses, DNA damage and repair, ABC pump mechanisms, mucin signals, mitochondrial dysfunction and apoptosis in patients with Cholelithiasis (CHL) and Choledocholithiasis (CHDL).

Materials and Methods: The relative fold-change in the mRNA expression levels of 73 genes were analysed using a gallstone-related qRT-PCR array in 10 control individuals, 24 CHL patients and 23 CHDL patients. Serum malondialdehyde levels was determined by thiobarbituric-acid reactive substance assays, and 8-hydroxy-2'-deoxyguanosine levels was determined by ELISA assays.

Results: Our results showed that peripheral whole blood gene expression profile strongly reflects tissue specific molecular signalling in gallstone pathogenesis. The present findings of altered gene expressions including antioxidant defence (CuZn-SOD, CAT), heat shock protein (HSP70), DNA repair (MLH1 and RAD18), pro-apoptotic (P53, BAX, Cyt-c and Caspase 3), ABC transporter (ABCB1, ABCC2, and LRP1) and Mucin signals (MUCIN4, MUCIN5AC and MUCIN5B) point out to DNA damages via oxidative stress as well as deteriorating ABC types pump mechanisms and mucin signals in CHL and CHDL patients. Our findings may also suggest that activation of mitophagy activator, DRP1/hFIS1/PINK1 axis induced by oxidative stress and DNA damage may have a role in the pathogenesis of CHL and CHDL.

Conclusion: Our results indicate that a blood-based gene expression signature has promising accuracy for monitorize pathogenesis of disease in CHL patients, CHDL patients and unaffected controls.

Introduction

Cholelithiasis (CHL) is one of the most prevalent gastrointestinal diseases leading to surgical intervention worldwide [1]. The direct and indirect results of gallbladder disease represent a cost of billions of dollars, causing a major health burden that increases every year [2,3]. Most patients are diagnosed when gallstones are discovered during an abdominal ultrasound that is being performed for another reason [4]. Increased mortality has been reported in patients with CHL associated with cardiovascular disease and cancer [5,6]. CHDL is a symptomatic gallstone disease characterized by the presence of gallstones in the common bile duct (CBD). It constitutes approximately 10 percent of the operated CBD patients and is one of the main causes of biliary obstruction. CHDL is prolonged, and some patients develop severe gallstone pancreatitis and gallbladder cancer, which can be life-threatening [7,8]. Therefore, the early diagnosis and disease prognosis of both diseases are important for the quality of life of the patient.

Recent molecular studies have focused on the aetiology of CHL and CHDL, inflammation, oxidative stress, epithelial tissue progression, reductions in proliferation, calcium metabolism, and mucin, cyclooxygenase (Cox)-2 pathways, and ATP-binding cassette (ABC) protein activities. One of the mechanisms of CHL that is caused by hypercholesterolemia is the induction of inflammatory changes in the gallbladder epithelium [9]. Inflammatory processes

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lead to the formation of reactive oxygen species (ROS) that affect cell function. ROS not only cause significant oxidative stress in gallbladder cells but also lead to the breakdown of lipids and the disruption of the cell wall structure, in particular by lipid peroxidation, and lead to an increase in the risk factors and the formation of the nuclei that form stones [10,11]. The gallbladder is capable of binding mucin lipids and bile pigment. The healthy epithelial layer of gallbladder expressed Mucin 2, 3, 4, 5B and 5AC genes. Both MUC4 and MU5AC mRNA have been found to be increased in cholangiocarcinoma and dysplastic cells in intrahepatic bile ducts containing stones. Mucin gel is believed to increase gallstone formation by supporting the deposition and aggregation of cholesterol crystals by binding to bile lipids. In addition, CHL and CHDL contribute to the formation of a reverse cholesterol transport mechanism, which plays a role in the transport of ABC transporters, the transport of cellular cholesterol to the cholesterol receptors, as well as cholesterol homeostasis in the gallbladder wall and gallbladder lumen, which are reported to play an important role in the formation of gallbladder stones [12-14].

Gallstone pathogenesis has been studied with varied molecular markers at tissue level. However, there is no comprehensive study on the effects of tissue-specific molecular signalling in the blood of gallstone patients, and blood based molecular signatures of the disease. Previous studies have propounded transcriptional profiling in peripheral blood as a good alternative for identification of disease activation. Potentially, gene expression profiles of peripheral blood could exhibit prior or progressive disease states and thus present valuable markers, with predicting treatment impact or a patient's prognosis. Different molecular signals in the aetiology of the disease can be accepted as biosensors, is influenced by disease-specific environmental factors, such as surrounding body fluids and all effector molecules therein, and may be useful in the diagnosis and prognosis. The aim of this study was to investigate the blood-based gene expression profiles that reflect disease-specific pathogenic mechanisms of gallstones, such as antioxidant defence, heat-shock responses, DNA damage and repair, ABC pump mechanisms, mucin signals, mitochondrial dysfunction and apoptosis, in CHL and CHDL patients.

Materials and Methods

Study population

All patient samples were obtained from the Trakya University Hospital between May 2017 and November 2018. Patients were preoperatively examined and selected for our study on the basis of the criteria given in Figure 1. Additionally, 10 samples were collected for qRT-PCR (quantitative reverse transcription PCR) validation. In total, 10 control, 24 CHL and 23 CHDL blood samples were used in the current study (Figure 1). Informed consent was obtained from all the patients, and the study was approved by the Ethics Committee of the Trakya University Medical Faculty (TUTF_BAEK-2017/261).

Blood sample collection and analysis

Intravenous blood samples were collected at hospital admission, before 24 hours from surgery. Samples were drawn into 5-cc K2EDTA or 5-cc sodium heparin vacutainers. The EDTA blood samples were directly placed into a liquid nitrogen tank for RNA isolation. Whole blood that was collected in heparin tubes kept at room temperature for 30 minutes and centrifuged at 2000 g for 10 minutes. Sera were then aliquoted into three (1–2 ml) aliquots and were frozen at −80 °C until subsequent analysis.

Determination of malondialdehyde (MDA) and 8-hydroxy-2-deoxyguanosine (8-OhdG)

The MDA level in serum was determined by a thiobarbituric acid reactive substance assay, as described by Kheradmand et al. (2009). Serum 8-OhdG levels were detected with an OxiSelect™ Oxidative DNA Damage ELISA Kit according to the manufacturer's protocol using a Thermo Multiscan Go multiplate reader spectrophotometer.

RNA isolation and complementary DNA (cDNA) synthesis

The TRIzol (Invitrogen, USA) reagent combine with PureLink™ RNA Mini Kit (Invitrogen, USA) were used to extract total RNA from whole blood. Purity control and RNA adjustment was performed with OPTIZEN NanoQ microvolume photometer. cDNA was synthesised using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) from 100 ng total RNA, and the concentrations were adjusted using water (Sigma, W4502). cDNA synthesis was performed using an Applied Biosystems® ProFlex™ PCR System (step 1: 25 °C, 10 min; step 2: 37 °C, 120 min; and step 3: 85 °C, 5 min). The cDNA was stored at −20 °C for subsequent analysis.

Quantitative real-time PCR (qRT-PCR) analysis

The expression levels of antioxidant system proteins, heat shock proteins (HSPs), DNA repair proteins, ABC and multidrug resistance cassette proteins, mitochondrial dysfunction proteins and mucin genes were determined in blood collected from controls, CHL patients and CHDL patients by qRT-PCR using PowerSYBR® Master Mix (Life Technologies, USA) and an ABI Quant studio 6 Real-Time PCR system with specific primers (additional information is given in Suppl. 1). The relative gene expression levels, normalised with β-Actin mRNA level, were calculated by $2^{-\Delta\Delta CT}$ method (User Bulletin 2, Applied Biosystems, USA).

Statistical analyses

All demographic data, routine biochemical parameters, MDA, 8-OhdG levels, and the relative fold change in gene expression due to CHL and CHDL were compared among groups using a Mann-Whitney U test and ANOVA with Tukey's multiple range test according to group number. Statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, N.Y., USA), and $P \leq 0.05$ was considered significant. Correlations between gene expression levels in controls, CHL patients, CHDL patients were analysed using a bivariate correlation test with Pearson's

correlation coefficient and a 2-tailed test of significance at significance levels of $P \leq 0.05$ and 0.001. Molecular and ELISA data are represented as the mean ± standard error (SE), unless otherwise noted. The normality of each variable was assessed before the appropriate statistical test was used. Interaction between gene expressions and CHL/CHDL disease were analysed both principal component analysis (PCA) and Heatmap analyses. The statistical significance was set at $p \leq 0.05$ for all analyses. All data were analysed using SPSS Version 20 (IBM, USA), ArrayMining Analysis (Stanford, USA), and Stata Version 15 (StataCorp, TX, USA).

Results

Demographic and clinical characteristics

Of the 47 gallstone patients, 24 had CHL, and 23 had CHDL. The average age of the patients was 48.76±3.84 and 61.72±15.41 years for CHL and CHDL patients, respectively. Three patients in CHL and two patients in CHDL groups reported moderate alcohol use, and four patients in CHL and two patients in CHDL groups actively smoked 10-20 cigarettes a day. The average body mass index (BMI) was 27.28±1.07 and 29.70±1.16 for CHL and CHDL patients, respectively.

The expression level of antioxidant defence, DNA repair mechanism and heat-shock response genes

To evaluate the blood molecular oxidative stress levels in patients with CHL and CHDL, we detected the gene expression of antioxidant enzymes, such as CuZn-superoxide dismutase (SOD), Mn-SOD, CAT (catalase), glutathione-S-transferase (GST), GPx (Glutathione peroxidase), GS (Glutathione synthase) and iNOS (Inducible nitric oxide synthase) (Table 1). Significant differences in four of the seven antioxidant defence genes were identified in control

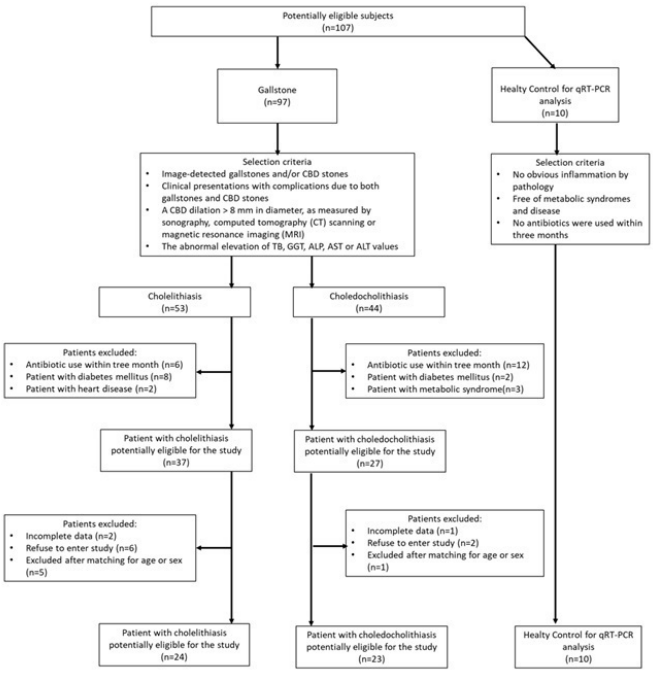
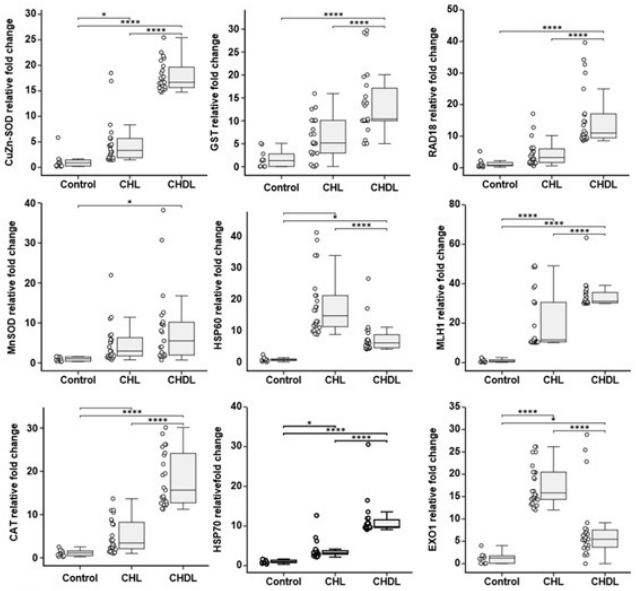


Figure 1. Flow diagram of the participants of the study.



All data were normalized with β-actin expression and given as relative to control. * indicating significantly different values were analysed by Oneway-ANOVA, Tukey HSD test.

Figure 2. The relative fold change determined by quantitative real-time PCR (qRT-PCR) analysis of CuZn-SOD, Mn-SOD, CAT, GST, HSP 60, HSP70, MLH1, RAD18, EXO1 genes in control, cholelithiasis (CHL) and choledocholithiasis (CHDL) populations.

patients compared to both CHL and CHDL patients. In addition, three of these four genes were significantly overexpressed in patients with CHDL compared to patients with CHL (Figure 2). The largest differences were observed for CuZn-SOD and CAT, which displayed a nearly 4.73-17.8- and 5.5-18.1-fold elevation in patients with CHL and CHDL, respectively, at hospital admission compared to controls. Preoperative blood Mn-SOD expression levels displayed a nearly 4.7-8.4-fold increase in those with gallstones and CBD stones, respectively, compared to those in the controls. The preoperative blood GST level was also significantly elevated in patients with both CHL and CHDL outcomes compared to that in controls (Figure 2). We further confirmed the oxidative stress via changes in the serum level of MDA, a biomarker of lipid peroxidation due to ROS. A similar pattern of elevation in the serum levels of MDA was exhibited in the CHL and CHDL groups (3.55- and 5.32-fold compared to the control group, respectively), which supported our molecular data. We investigated DNA damage via changes in the expression of eight genes belonging to DNA repair pathways, and serum 8-OHdG levels. Three of the eight DNA damage markers were significantly different between the control and experimental groups (Table 1). While the highest increase (17.6-fold compared to the control) in the DNA mismatch repair (MMR) genes was observed for the gene EXO1 in patients with CHL, the MMR gene MLH-1 (37.9-fold increase compared to the control) and the gap-filling DNA repair gene RAD-18 (18.06-fold increase compared to the control) were also overexpressed, particularly in patients with CHDL. Therefore, the last two DNA repair genes significantly differentiated the CHL and CHDL groups from

Table 1. Relative fold change qRT-PCR analysis of gallstone related pathways genes in control, Cholelithiasis (CHL) and Choledocholithiasis (CHDL) population.

Genes	Control	CHL	CHDL	P	Genes	Control	CHL	CHDL	P
Antioxidant System					Mitochondrial Dysfunction				
CuZn-SOD*	1.28±0.53^{a}	4.73±0.90^{b}	17.88±0.60^{c}	.000	Drp1*	1.75±0.92^{a}	4.66±0.35^{b}	17.70±4.21^{c}	.001
Mn-SOD*	1.01±0.16^{a}	4.78±0.97^{ab}	8.49±1.95^{b}	.014	hFIS1*	1.03±0.17^{a}	4.08±0.26^{b}	12.62±2.66^{c}	.000
CAT*	1.12±0.23^{a}	5.50±1.60^{b}	18.16±1.30^{c}	.000	Mff	1.13±0.28^{a}	1.39±0.33^{a}	1.01±0.38^{a}	.724
GST	1.20±0.50^{a}	9.10±2.64^{ab}	12.54±3.71^{b}	.117	MID	1.14±0.27^{a}	0.85±0.12^{a}	1.82±0.53^{a}	.156
GPx*	0.95±0.11^{b}	0.45±0.07^{a}	0.62±0.13^{ab}	.030	Mfn1	1.48±0.70^{a}	3.38±0.80^{a}	4.03±0.84^{a}	.203
GS	1.12±0.21^{a}	1.88±0.50^{a}	2.36±0.45^{a}	.310	Mfn2*	1.10±0.19^{a}	2.40±0.55^{ab}	3.90±0.77^{b}	.038
iNOS	0.93±0.22^{a}	5.24±1.39^{b}	2.82±0.81^{ab}	.066	OPA1*	0.95±0.11^{a}	7.62±0.90^{b}	20.57±1.55^{c}	.000
Heatshock Protein Families					PINK1*	1.22±0.31^{a}	5.74±0.67^{b}	18.93±0.86^{c}	.000
HSP27	1.01±0.20^{a}	3.74±0.91^{a}	4.26±1.18^{a}	.179	BNIP3	0.97±0.15^{a}	2.09±0.27^{b}	2.21±0.35^{b}	.054
HSP60	1.05±0.21^{a}	18.25±1.97^{c}	7.89±1.07^{b}	.000	NIP3X*	1.08±0.25^{b}	0.25±0.07^{a}	0.07±0.04^{a}	.000
HSP70	0.96±0.13^{a}	4.20±0.58^{b}	11.47±0.94^{c}	.000	NRF2A*	0.94±0.10^{a}	4.96±1.13^{ab}	8.37±1.58^{b}	.007
HSP90	1.40±0.44^{a}	3.73±0.90^{a}	3.88±0.60^{a}	.144	PPAR	0.98±0.15^{a}	4.02±0.53^{a}	12.26±9.76^{a}	.513
Gene Repair					ATP-binding cassette transporters (ABC transporters)				
MLH1*	0.98±0.25^{a}	24.85±5.42^{b}	37.98±4.05^{b}	.000	ABCB1*	1.12±0.49^{ab}	0.18±0.11^{a}	1.99±0.38^{b}	.000
MSH2	0.96±0.11^{a}	1.69±0.31^{a}	2.05±0.55^{a}	.343	ABCC1*	0.99±0.15^{b}	0.01±0.00^{a}	0.06±0.04^{a}	.000
XRCC1	1.38±0.38^{a}	3.03±0.61^{a}	3.28±0.94^{a}	.345	BCRP*	1.18±0.31^{b}	0.19±0.04^{a}	0.20±0.05^{a}	.000
XRCC3*	1.47±0.55^{b}	0.56±0.15^{a}	2.22±0.19^{c}	.000	ABCC2*	0.95±0.10^{a}	3.10±0.20^{b}	6.79±0.25^{c}	.000
RAD18*	1.35±0.48^{a}	4.51±0.81^{a}	18.06±3.03^{b}	.000	LRP1*	1.09±0.22^{a}	1.73±0.10^{a}	4.92±0.55^{b}	.000
EXO1*	1.31±0.38^{a}	17.66±0.93^{c}	9.02±2.36^{b}	.000	Mucin Signals				
NEIL2	1.12±0.29^{a}	1.79±0.26^{a}	3.23±1.26^{a}	.292	MUC2	1.08±0.21^{a}	1.28±0.15^{a}	2.85±0.90^{a}	.107
SMUG1	1.05±0.18^{a}	0.74±0.12^{a}	1.68±0.65^{a}	.283	MUC3*	1.00±0.16^{a}	1.83±0.33^{ab}	3.69±0.90^{b}	.030
Cell Cycle Arrest					MUC4*	0.95±0.12^{a}	2.49±0.32^{b}	21.05±0.45^{c}	.000
P21*	1.02±0.20^{b}	0.77±0.31^{ab}	0.16±0.07^{a}	.047	MUC5B*	1.07±0.18^{a}	4.86±0.61^{b}	16.85±1.21^{c}	.000
P27*	0.92±0.18^{a}	1.85±0.18^{a}	14.16±2.55^{b}	.000	MUC5AC*	1.04±0.18^{a}	1.66±0.12^{a}	6.45±1.18^{b}	.000
Mitochondrial Apoptosis					Hipoxia and Angiogenesis				
PUMA*	1.11±0.23^{a}	0.94±0.12^{a}	3.82±0.62^{b}	.000	EGFR*	1.18±0.31^{a}	7.43±1.03^{b}	18.49±2.19^{c}	.000
NOXA*	0.94±0.10^{a}	1.93±0.26^{a}	4.26±0.45^{b}	.000	IFNAR1	1.18±0.36^{a}	4.46±1.78^{a}	1.85±0.51^{a}	.209
BID	0.97±0.13^{a}	0.87±0.10^{a}	1.39±0.32^{a}	.226	VEGF*	1.00±0.17^{a}	2.65±0.40^{a}	9.10±1.38^{b}	.000
BAK	1.05±0.19^{a}	0.96±0.12^{a}	2.10±0.64^{a}	.129	VEGFR*	1.56±0.42^{a}	6.98±1.77^{b}	15.55±1.07^{c}	.000
PI3K*	1.39±0.49^{a}	3.30±0.22^{b}	3.16±0.24^{b}	.000	NFKβ*	1.01±0.15^{b}	0.05±0.01^{a}	0.07±0.04^{a}	.000
AKT*	1.03±0.20^{a}	1.46±0.11^{a}	8.12±1.73^{b}	.000	COX2	0.97±0.13^{a}	1.90±0.23^{b}	1.80±0.24^{b}	.056
P53*	0.98±0.14^{a}	3.23±0.84^{a}	11.90±2.22^{b}	.000	JUNB*	1.06±0.21^{a}	0.96±0.13^{a}	5.80±0.24^{b}	.000
BAX*	2.32±1.50^{a}	9.05±0.81^{b}	15.30±2.08^{c}	.000	HIF1α*	0.99±0.18^{a}	0.96±0.06^{a}	4.25±0.37^{b}	.000
BCL2	0.96±0.12^{a}	1.07±0.29^{a}	0.39±0.30^{a}	.201	HIF1β*	1.01±0.16^{b}	0.14±0.03^{a}	0.07±0.05^{a}	.000
BCLXL*	0.99±0.14^{b}	0.26±0.03^{a}	0.44±0.10^{a}	.000	MMP3	0.97±0.15^{a}	1.39±0.12^{a}	1.55±0.24^{a}	.214
XIAP	0.98±0.16^{a}	1.00±0.12^{a}	0.93±0.16^{a}	.938	MMP9*	1.08±0.20^{a}	1.68±0.28^{a}	10.85±0.31^{b}	.000
CYT-C*	1.09±0.35^{a}	14.54±3.65^{b}	31.15±1.89^{c}	.000	MAPK7	1.13±0.27^{a}	1.61±0.20^{ab}	2.62±0.58^{b}	.078
APAF1	1.81±0.30^{a}	5.51±1.22^{ab}	7.74±1.64^{b}	.056	MAPK3*	0.97±0.13^{b}	0.05±0.02^{a}	0.18±0.12^{a}	.000
CAS3	1.06±0.22^{a}	5.23±0.62^{b}	10.72±0.79^{c}	.000					
CAS8*	0.98±0.15^{a}	2.69±0.32^{ab}	5.36±1.67^{b}	.063					
mTOR	0.99±0.14^{a}	3.02±0.66^{ab}	4.43±1.35^{b}	.150					
IκBα*	1.10±0.25^{a}	3.94±0.27^{b}	4.01±0.54^{b}	.000					

All data were normalized with β-actin expression and given as relative to control; Data represented mean±SE *Different letter in the rows indicating significantly different values were analysed by one-way ANOVA and Tukey HSD test.

the control group. We further confirmed the differences in the DNA damage pathways via changes in the serum level of 8-OHdG. While there was a significant increase in serum 8-OhdG levels in the disease group compared to the control group (P=0.066), there were no statistically significant differences between the CHL and CHDL groups; (2.21-fold-2.61 fold, respectively) however, a slightly higher level was observed in patients with CHDL compared to patients with CHL. In our study, HSP27, HSP60, HSP70 and HSP90 gene expression levels were examined in the various groups. We found significant differences in only HSP70 among these four genes from HSP families in the control group versus the disease groups and in the CHL versus the CHDL groups (Figure 2).

Table 2. The eigenvectors and factor loadings values for the first two factors resulting from PCA.

		Princible Component Analysis				Heatmap Analysis					Multiple Comparison Test	
		Eigenvectors		Factor loadings		Log (x+1) adjusted			Control vs Gallstone		Cholelithiasis vs Choledocholithiasis	
Pathway	Gene	F1	F2	F1	F2	PC (gene vs OC)	F-statistic	Q-value	Pearson r	p value	Pearson r	p value
Antioxidant defence	CuZn_SOD	0.154	-0.078	0.877	-0.262	0.88	108.61	1.50E-19	0.681	5.55E-05	0.841	1.39E-09
	CAT	0.159	-0.081	0.907	-0.174	0.84	73.59	5.20E-16	0.628	1.66E-03	0.772	2.12E-06
HeatShock Response	HSP70	0.154	-0.077	0.879	-0.259	0.91	128.94	4.70E-21	0.765	4.26E-08	0.826	8.77E-09
DNA repair	MLH1	0.129	-0.124	0.739	-0.420	0.83	118.93	2.50E-20	0.892	1.30E-16	0.521	1.73E+00
	RAD18	0.155	-0.009	0.882	-0.030	0.80	53.02	2.30E-13	0.612	4.26E-04	0.727	7.11E-05
Mitochodrial Disfunction	Drp_1	0.155	-0.083	0.887	-0.078	0.87	87.89	1.40E-17	0.729	1.29E-06	0.842	1.19E-09
	hFIS_1	0.159	-0.095	0,906	-0.218	0.90	112.80	7.80E-20	0.772	2.09E-08	0.813	3.76E-08
	OPA_1	0.161	-0.100	0.920	-0.337	0.93	205.82	1.60E-25	0.852	4.08E-14	0.832	4.24E-09
	PINK_1	0.153	-0.107	0.875	-0.363	0.94	208.53	1.60E-25	0.774	1.72E-08	0.911	6.34E-15
Mitochondrial Apoptosis	P53	0.125	-0.077	0.713	-0.124	0.75	44.37	5.30E-12	0.385	3.09E-03	0.702	3.90E-04
	BAX	0.146	-0.096	0.832	-0.222	0.77	60.18	2.30E-14	0.807	3.41E-10	0.517	1.97E+00
	CYT-C	0.137	-0.123	0.783	-0.417	0.88	108.70	1.50E-19	0.823	4.12E-11	0.720	1.19E-04
	CAS_3	0.156	-0.080	0.890	-0.136	0.84	67.57	2.50E-15	0.759	8.19E-08	0.644	1.06E-02
ABC transporters	ABCB1	0.084	0.033	0.481	0.112	0.79	26.55	1.40E-13	-0.228	8.74E-02	0.844	9.10E-11
	ABCC2	0.161	-0.080	0.919	-0.269	0.94	171.00	8.40E-24	0.794	1.75E-09	0,859	1.17E-10
	LRP1	0.157	-0.100	0.898	-0.097	0.83	70,62	1.20E-15	0.580	2.26E-02	0.857	1.47E-10
Mucin signals	MUC_4	0.150	-0.169	0.858	-0.232	0.90	291.92	5.90E-29	0.530	2.26E-02	0.910	8.05E-15
	MUC5B	0.159	-0.074	0.910	-0.249	0.91	140.96	6.40E-22	0.734	8.46E-07	0.847	5.89E-10
	MUC5AC	0.160	-0.084	0.915	-0.149	0.81	68.60	1.90E-15	0.531	2.12E-01	0.844	9.76E-10

Adjusted Heatmap analysis: PC score and Q value and Person Correlation Analysis: Correlation score, P value result of blood gene expressions in CHL and CHDL populations.

Mitochondrial apoptosis and dysfunction

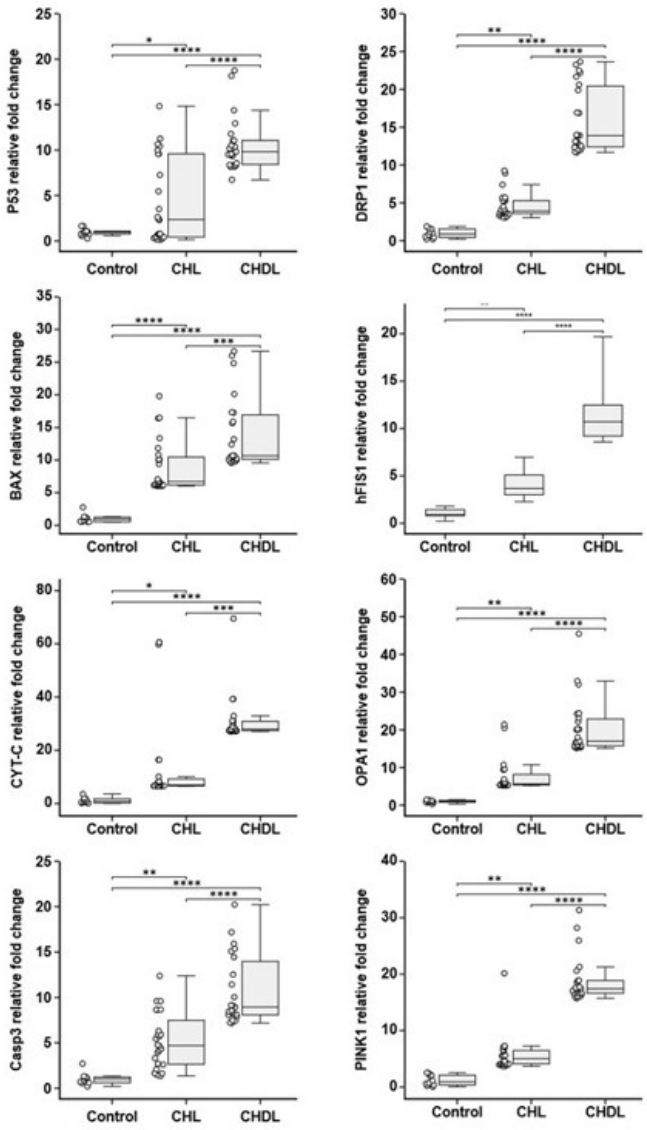
The relative expression levels of four of the 17 apoptosis markers were significantly different between the control and experimental groups (Table 1). In addition, the expression levels of the BAX (9.1-15.3-fold), CYT-C (14.5-31.1-fold), and Caspase 3 (5.2-10.7-fold) genes, which belong to the intrinsic apoptosis pathway, were significantly different in CHL and CHDL groups (Figure 3). Figure 3 also shows the expression profiling of the mitochondrial fission markers DRP1 and FIS1 and the mitophagy and apoptosis markers PINK and OPA1, which were considered a functional group of mitochondrial dysfunction, as analysed by the qRT-PCR array. Compared to the expression in the control group, all of these genes were significantly upregulated in CHL patients (4.1- 7.62-fold) and were significantly overexpressed in CHDL patients (12.6-20.5-fold) (Figure 3).

ABC transporters and mucin signals

Eleven genes associated with ABC transporters and mucin signals were analysed. Genes associated with both ABC transporters (ABCC2 and MXR) and mucin signals (MUC3, MUC4 and MUC5B) upregulated in the CHL and CHDL groups compared to the control group. However, genes with decreased expression in the CHL and CHDL groups compared to the control group were dedected in functional groups associated with ABC transporters (ABCB1, ABCC1 and BCRP). In comparing the individual gene expression of CHL versus CHDL patients, the expression of three ABC transporter genes (ABCB1, ABCC2 and LRP1) and three mucin signal genes (MUCIN 5AC, MUCIN 5B and MUCIN 4) remained positively associated with CHDL (Figure 4, Table 1).

Genes and CHL/CHDL interactions

Three factors were retained for the PCA, and their cumulative percentages were calculated as 39.80, 52.86 and 63.13% for the control and experimental groups, respectively. The first two factors involved the peripheral blood-based gene expression, which accounted for 92.63% of the cumulative variance, and were plotted against each other. The eigenvalues, variance, cumulative variance, eigenvectors and factor-loading values of the two PCs are shown in Table 2. In the coordinate systems, while almost all patients in the CHDL group and control group shared two different poles on X and Y, CHL patients showed a close distribution near the centre. The CHDL group, which was clearly different from both the control and CHL groups, was located in the positive x and negative y fields of the coordinate systems, under the effect of the positive vectors of the first PC and the negative vector of the second PC, which included functional genes involving ABC transporters (ABCB1, ABCC2, and LRP1), mitochondrial dysfunction (DRp1, hFIS1, OPA1 and PINK1), mitochondrial apoptosis (P53, BAX, Cyt-C and CASP 3), mucin signals (MUC4, MUC5AC, and MUC5B), oxidative defence (CuZn-SOD and CAT) and the heat-shock response (HSP70) (Figure 5A). To further evaluate interaction characteristics of our gene expression with healthy and CHL/CHL populations, we performed heatmap analy-



All data were normalized with β-actin expression and given as relative to control. * indicating significantly different values were analysed by Oneway-ANOVA, Tukey HSD test.

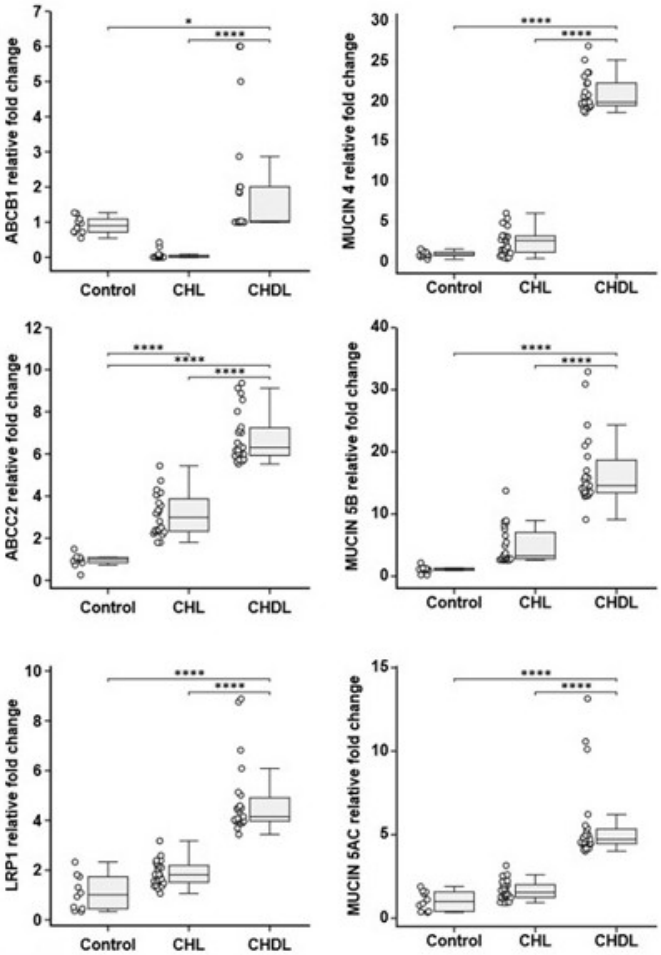
Figure 3. The relative fold change determined by quantitative real-time PCR (qRT-PCR) analysis of P53, BAX, CYT-C, Caspase 3, HFIS1, DRP1, PINK1 and OPA1 genes in control, cholelithiasis (CHL) and choledocholithiasis (CHDL) populations.

sis. Figure 5B shows the heatmap of the array experiment, which was created by the Z-score, hierarchical clustering and Bayesian properties of the gene expression levels to facilitate the visual analysis of the data. Significant right-left asymmetry was observed, indicating that there were significant changes in the gene expression among the control and experimental groups, and three hotspot areas were clearly detected in the heatmap. The most effective parameters were ABCC_2, PINK1, OPA1, HSP70, MUC5b, hFIS1, MUC4, and CuZn-SOD, which had the highest PC scores, and they shared the first five ranks based on the q-value of the heatmap analyses (Table 2).

Discussion

Our analyses were designed to confirm a possible molecular gene expression signature using peripheral blood microarray data obtained from healthy controls, CHL patients and CHDL patients in combination with machine learning algorithms. In this exploratory study using the gene expression array data of genes involved in oxidative stress, DNA damage, heat-shock response, ABC transporters, Mucin signals, mitochondrial dysfunction and apoptosis pathways, which play primary and secondary roles in CHL and CHDL pathophysiology, we identified a blood-based gene expression signature that reliably identified healthy adults and patients with CHL and CHDL.

Both CHL and CHDL are multifactorial diseases based on a complex interaction of environmental and genetic factors. Recent evidence suggests that gallstone disease is associated with inflammation, hypoxia, oxidative stress and DNA damage, with profound effects on cellular and gallbladder physiology and many pathophysiological consequences [1,15,16]. In gallstone disease, gastrointestinal



All data were normalized with β-actin expression and given as relative to control. * indicating significantly different values were analysed by Oneway-ANOVA, Tukey HSD test.

Figure 4. The relative fold change determined by quantitative real-time PCR (qRT-PCR) analysis of LRP1, ABCC2, ABCB1, MUCIN4, MUCIN 5B and MUCIN 5AC genes in control, cholelithiasis (CHL) and choledocholithiasis (CHDL) populations.

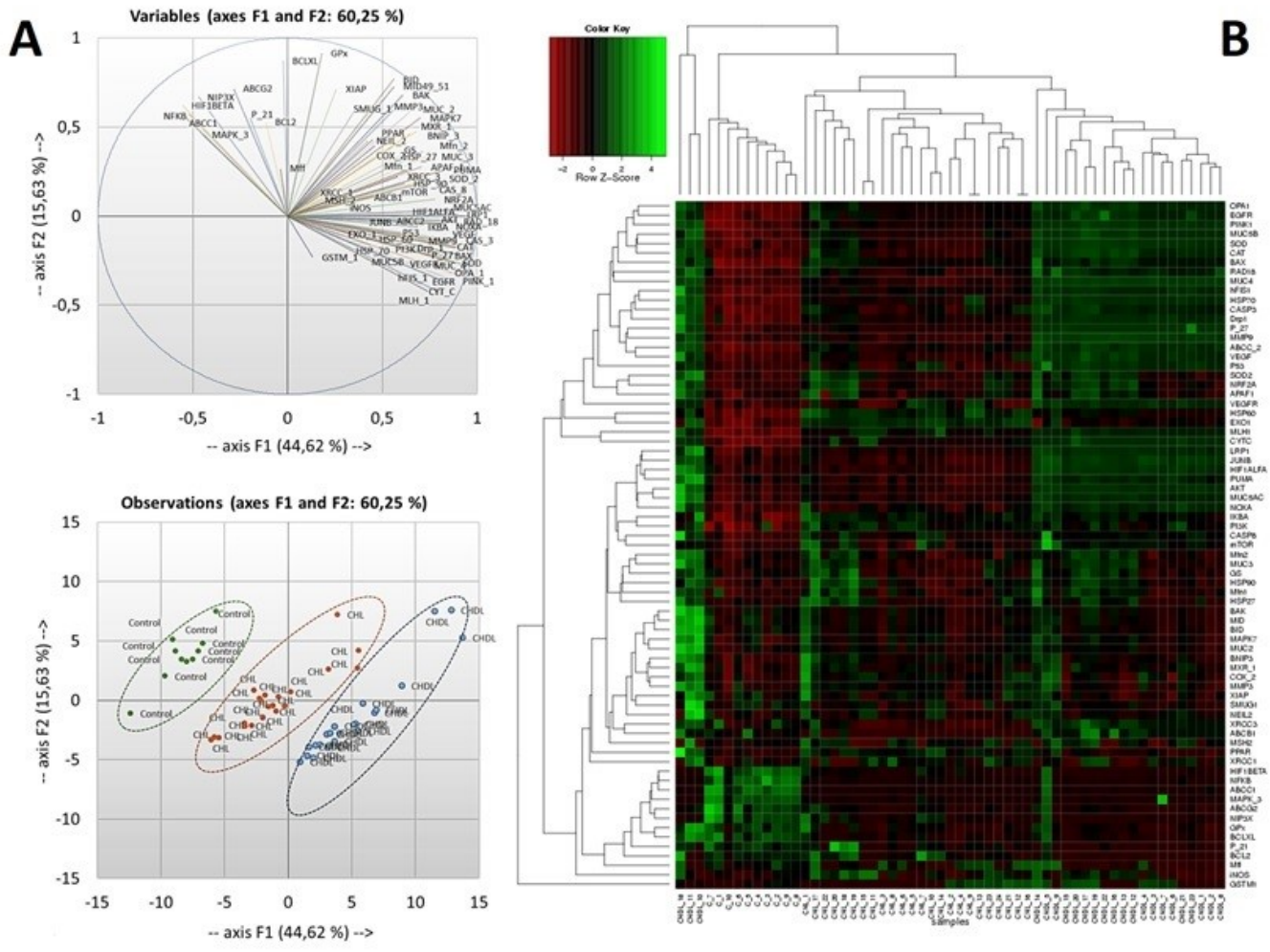


Figure 5. A) Scatter plot for control, cholelithiasis (CHL) and choledocholithiasis (CHDL) population and vectors (73 genes) distributed on the coordinate system of the first two factors resulting from principal component analysis. PCA analysis of differentially expressed genes belong to gallstone related pathway with reasonable expression levels and amounts demonstrated a clear separation between the control, CHL and CHDL groups. B) Heat map analysis using 73 gene expressions levels of Control, CHL and CHDL groups. All data Log (x +1) transformed and eBayes and Pearson correlation options were used hierarchical clustering and correlation analysis, respectively.

pro-oxidant and antioxidant molecules not only react to a number of bile components, such as bile acid, cholesterol, and bilirubin [17], but also directly attack the protein and transcriptional products of mucosal epithelial cells [18] and smooth muscle layer cells [19], consequently triggering oxidative stress. Indeed, recent evidence suggests that ROS have an important role in gallstone formation by accelerating cholesterol crystal formation and markedly reducing cholesterol nucleation times [20] . In addition, ROS trigger lipid peroxidation and produce MDA, which enhances inflammation by activating chemotactic factors and granulocyte-attracting molecules [21] and induces mucin secretion [18]. Antioxidant defence signals, such as SOD, catalase, GST, etc., are rapid and effective protective mechanisms against ROS; for this reason, they are widely used in both the development of molecular biomarkers and the study of basic pro-oxidant/antioxidant processes [22,23]. Consistent with a previous study, we observed that both CHL and CHDL induced lipid peroxidation, as shown by the increase in MDA levels. Additionally, in this study, the relative expression levels of cytosolic CuZn-SOD, mitochondrial Mn-SOD, CAT and GST were significantly upregulated in the CHL and CHDL groups. The gallstone-induced upregulation of SOD1 was also found in the plasma of surgical gallstone patients compared to cancer patients [24], and high levels of SOD, CAT and GST activity were also reported in the gallbladder mucosa of gallstone patients [25]. In the current study, we identified 5 genes that were significantly upregulated and one gene that was significantly downregulated in the gallstone groups in qRT-PCR analyses. However, only CuZn-SOD and CAT yielded significantly higher Pearson correlation coefficients in the CHL and CHDL groups.

In gallstone disease, the gallbladder and CBD microenvironment, such as ROS, damaged protein lipids and especially, bile salts, are closely related to the DNA damage process. Bile acid and bile salts are important causes of DNA damage with both mechanical and detergent proper-

ties [26]. In gallstone pathology, oxidative stress, as well as the micro-environmental stress during the gallstone formation process in the smooth muscle layer and mucosal layer, are combined with the mechanical damage caused by gallstones, resulting in large amounts of DNA damage caused by bile salts in both the gallbladder and bile duct [26,27]. Consistent with previous studies [28-30], we observed a correlation between oxidative stress, which was dependent on disease activity, and DNA damage and a lower effectiveness of gene repair mechanisms in the CHL and CHDL groups, as shown by significantly higher serum 8-OHdG levels, despite the overexpression of the MLH1, RAD18 and EXO1 genes. In the gallbladder, 8-OHdG expression was mainly found in the nuclei of mucosal epithelial cells in areas of active inflammation. A previous study reported that 8-OHdG levels were sensitive biomarkers of oxidative DNA damage in gallbladder mucosa with chronic cholecystitis. Additionally, the significant expression of MLH1, a MMR gene, was observed in human pancreatic cancers and gallbladder carcinoma [31]. The EXO1 gene is a member of the RAD2 nuclease family and functions in DNA replication, strand-break repair and recombination; additionally, the RAD18 gene, which is located on human chromosome 3p24-p25, has an important role in post-replication repair (PRR) in several types of tissue and organs, and genetic polymorphisms of the EXO1 and RAD18 genes have been reported to determine the susceptibility to several cancers, including lung, oesophageal, and gallbladder cancers [32,33]. Significantly increased HSPs expression a responses to stress-specific protein damage in cells [34]. Thus, the increased stress-specific HSP70 induced by gallstone disease, as observed in our experiments, could be a potential biomarker for unfolded/misfolded proteins in the peripheral blood of patients with CHL and CHDL.

Increased oxidative damage and breaks or mistakes in nucleic acid chains after the disruption of antioxidant defence signalling or after unsuccessful DNA repair and HSP activation trigger mitochondrial dysfunction and then cell cycle arrest and apoptosis. Consistent with these findings, we showed that both CHL- and CHDL-impaired signalling triggers the activation of major mitochondrial fission and the mitophagy activator DRP1/hFIS1/PINK1 axis, as well as the apoptosis-related OPA1 gene. Furthermore, our results showed P27kip1 (G1/S phase arrest) was overexpressed and P53 was significantly activated in both the CHL and CHDL groups compared to in healthy individuals, and these effects depended on the mitochondrial apoptosis pathway. No studies were found investigating the genes from our study in the blood samples of CHL and CHDL patients. However, the relationship between mitochondrial dysfunction and metabolic syndrome [35], high cholesterol accumulation [36], bile acid toxicity [37] and impaired fatty acid metabolism [38], which are among the most important risk factors for gallstone diseases, have been well documented. Our results on the peripheral expression signatures of mitochondrial apoptosis signals are in agreement with the data reported by Sou et al. in 2004, in which they reported that different types of sterols (especially oxysterol) induce apoptosis, as detected by Hoechst 33342 and Mito Tracker Green probe staining and Cyt-c release in primary cultures of dog gallbladder epithelium. Mitochondrial dysfunction and intrinsic apoptosis signals caused by the damage of the mitochondrial membrane potential may be the main mechanisms that stimulate the bile acid and oxidative protein modification-induced apoptosis of hepatocytes in animal models and patients with CHDL [39,40].

ABC transporters and mucin signalling play an important role in the pathophysiology of gallstones. These genetic signals are responsible for the secretion of cholesterol, phospholipids, bile salts and bile pigment bilirubin contained in the bile [16], as well as for the balanced release of these factors into and out of the cell. In this process, ABCB4 acts as a phospholipid transporter, ABCB11 acts as a transporter, and heterodimers of ABCG5 and ABCG8 act as cholesterol transporters [1,41]. Almost all of the studies on the ABC transporter/gallstone relationship have been conducted by investigating single-nucleotide polymorphisms (SNPs), gene variations and gene mutations. In these studies, all of the mutations that led to the overexpression of these genes were found to be associated with gallstone disease [41,42]. In our study, ABC type carrier signaling in the control, CHL and CHDL groups was analysed by examining the ABCB1, ABCC1, ABCC2, MXR, BCRP and LRP genes. Our results are consistent with those from two previous studies in a mutant mouse model that demonstrated that ABCC2 overexpression is directly associated with gallstone formation [43] and a gene polymorphism study that demonstrated that LRP mutations in 214 gallstone patients were associated with gallstones [44]; additionally, while the ABCC2 and LRP genes were significantly overexpressed in CHL patients compared to the control group, the expression was significantly higher in the patients with CHDL than in the patients with CHL.

Mucin gel has the ability to bind lipids and bile pigment and plays an important role in the pathogenesis of gallstone disease by binding to biliary lipids and promoting cholesterol crystal precipitation and aggregation; this interaction causes the formation of several types of pigment and cholesterol stones [45,46]. In healthy individuals, MUC2, MUC3, MUC4, MUC5B, and MUC5AC, the main genes in the mucin pathway, were expressed by gallbladder epithelial cells; in response to stress conditions, MUC4, MUC5B and MUC5AC were rapidly and significantly expressed in the microenvironment [47,48]. A previous study demonstrated that there is a significant correlation between mucin 2, MUC5AC, and Mucin 4 secretion and inflammatory processes (such as increased levels of Interleukin-1β and TNF), several types of bile stones and cholangiocarcinomas [49]. Consistent with previous findings, in our study, Mucin 5AC, Mucin 5B and Mucin 4 expression were significantly higher in the CHDL population than in both the CHL and control populations.

To the best of our knowledge, this study is the first to report the basal and disease-associated levels of gallstone-related gene expression in the blood, which included genes associated with antioxidant defence, the heat-shock response, mitochondrial dysfunction, apoptosis, ABC transporters and Mucin pathways. Additionally, this was the first study to examine the interaction between blood gene expression signatures and gallstone pathogenesis in

healthy individuals and in CHL and CHDL events in a cohort of gallstone patients. Our results showed that peripheral whole blood gene expression profile strongly reflects tissue specific molecular signalling in gallstone pathogenesis. Our findings may also suggest that the strongly active mitophagy activator DRP1/hFIS1/PINK1 axis induced by oxidative stress and DNA damage may have a role in the pathogenesis of CHL and CHDL. In conclusion, our results indicate that a blood-based gene expression signature has promising accuracy for monitorize pathogenesis of disease in CHL patients, CHDL patients and unaffected controls. Additionally, the 19 differentially expressed genes belong to gallstone-related signalling pathways may encourage the development of an important method of blood based clinical biomarker discovery, offering high diagnostic accuracy for detecting both CHL and CHDL.

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Ethics approval

This study was approved by the Ethics Committee of the Trakya University Medical Faculty (TUTF_BAEK-2017/261).

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