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1st Faculty of Medicine

PhD thesis summary

The Heme Catabolic Pathway in Chronic Hepatitis C

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The full text of the thesis will be available at least five days before the PhD defense at the Department of Science and Research and International Relations of the 1st Faculty of Medicine, Charles University in Prague.

Contents

Abstract:

This thesis focuses on the importance of the heme catabolic pathway in chronic hepatitis C (HCV). The aim is mainly to investigate, whether expresion/activity of key enzymes of the heme catabolic pathway, heme oxygenase (HMOX) and biliverdin reductase (BLVRA) in the liver and blood (*study A*) or promoter variations of *HMOX1* and UDP-glucuronosyltransferase (*UGT1A1*) (*study B*) may be associated with the progression of fibrosis and may also predict antiviral treatment outcome in patients chronically infected with HCV.

We set up a new sensitive method to quantify HMOX activity by reduction gas chromatography. We developed and extensively validated RealTime PCR assay for *HMOX* and *BLVRA* expression in the liver and peripheral blood leucocytes (PBL). The (GT)n and (TA)n dinucleotide variations in *HMOX1* and *UGT1A1* gene promoters, respectively, were determined by fragment analysis.

No association was detected between either expression of *HMOX/BLVRA* or the *HMOX1/UGT1A1* promoter variants and the individual histological stages of liver disease in the HCV positive patients. A marked difference in *BLVRA* expression in PBL between the sustained responders (SVR) and patients with treatment failure (NVR) was detected before antiviral treatment and during the follow-up. Our data suggests, that *BLVRA* basal expression in PBL may be an independent predictor of SVR.

Abstrakt:

Předkládaná práce se zabývá významem katabolické dráhy hemu u chronické hepatitidy C. Je zaměřena na objasnění možné asociace exprese/aktivity klíčových enzymů katabolické dráhy hemu, hemoxygenázy (*HMOX*) a biliverdinreduktázy (*BLVRA*) v jaterní tkáni a krvi (*studie A*) a polymorfismů *HMOX1* a UDP-glukuronosyltransferázy (*UGT1A1*) (*studie B*) s rozdílnými histologickými nálezy a rozdílnou účinností protivirové terapie.

Cílem práce bylo nalézt faktory, které by u pacientů s chronickou HCV infekcí mohly predikovat progresi jaterního poškození a efekt protivirové terapie před jejím zahájením.

Pro účely této studie jsme zavedli vysoce citlivou metodiku na stanovení aktivity HMOX v mononukleárech periferní krve (PBMC), která dosud nebyla k dispozici. Zavedli jsme a zvalidovali RealTime PCR metodiku na stanovení exprese *HMOX* a *BLVRA* jaterní tkáni a v periferních leukocytech (PBL). (GT)n a (TA)n variace promotoru genu pro *HMOX1* a *UGT1A1* jsme stanovili simultánní fragmentační analýzou, jejíž metodika byla v naší laboratoři nedávno vyvinuta a rovněž publikována.

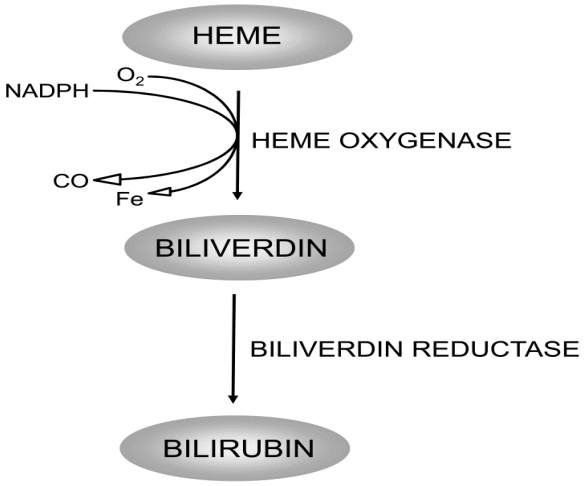
Pomocí uvedených metod jsme neprokázali asociaci exprese/aktivity enzymů katabolické dráhy hemu a polymorfismů *HMOX1* a *UGT1A1* s histologickou aktivitou v jaterní tkáni. Prokázali jsme však významné rozdíly v expresi *BLVRA* v PBL mezi pacienty se setrvalou virologickou odpovědí (SVR) a pacienty bez virologické odpovědi (NVR), a to jak před zahájením terapie, tak i v jejím průběhu. Multivariační analýza potvrdila, že bazální expresi *BLVRA* v PBL je nezávislým prediktorem SVR.

1. Introduction

Hepatitis C virus (HCV) infection represents one of the leading causes of chronic hepatitis worldwide, resulting in progression into fibrosis, cirrhosis and hepatocellular carcinoma in a significant number of HCV-infected patients [1]. The HCV prevalence is estimated to be 3% worldwide [2] and 0.2 - 0.5 % in the Czech Republic with predominance of genotype 1 (79.3%) and 3 (19.7%) [3,4].

Although HCV is mainly hepatotropic, there is also evidence that it can replicate in the peripheral blood mononuclear cells (PBMC) of patients with chronic HCV infection [5]. Oxidative damage has been hypothesized to play a role in HCV-induced liver disease, with reactive oxygen and nitrogen species (RONS) generated from HCV-infected hepatocytes, and infiltrating the immune cells [6,7]. HCV might not only increase RONS production, but also downregulate expression of certain antioxidant genes, including heme oxygenase (HMOX) [8]. HMOX catalyzes the degradation of the pro-oxidative heme to biliverdin, carbon monoxide (CO), and iron Biliverdin is then subsequently reduced to bilirubin by biliverdin reductase (BLVR) (**Figure 1**).

Figure 1. The heme catabolic pathway



Biliverdin, bilirubin, and CO exert numerous biological functions, including anti-oxidative and anti-inflammatory effects, as well as the modulation of cell proliferation and apoptosis [9,10,11]. Two HMOX isoforms have evolved, which include HMOX1 (OMIM*141250), an inducible isoenzyme, and HMOX2 (OMIM*141251), a constitutive isoform. Both catalyze the same reaction, but are differentially regulated, and play different roles in protecting tissues against oxidative injuries [12]. An accumulating body of evidence suggests that *HMOX1* overexpression contributes to cellular response against oxidative stress [13], and might have strong anti-fibrotic, as well as an anti-apoptotic potential within the liver tissue [14,15]. *HMOX1* induction *in vitro* has recently been shown to decrease HCV replication [16]. On the other hand, reduced *HMOX1* expression has been reported in the liver tissue of patients with chronic hepatitis C [8]; although under *in vitro* conditions hepatic *HMOX1* overexpression in the presence of HCV proteins has been reported by other authors [17]. Hepatic bilirubin UDP-glucuronosyl transferase (UGT1A1, OMIM *191740), the other important enzyme in heme catabolic pathway, is responsible for conjugation of bilirubin with two molecules of glucuronic acid, facilitating thus its elimination into to biliary system. Congenital reduction in bilirubin glucuronidation results in mild, chronic, fluctuating unconjugated hyperbilirubinemia (Gilbert's syndrome)[18].

A number of recent studies have shown that specific promoter variations in both the *HMOX1* and *UGT1A1* genes are associated with various pathological conditions (for review see [18]). The *HMOX1* gene promoter contains a highly polymorphic dinucleotide GT repeats (ranging from 11 to 42) which are responsible for the regulation of *HMOX1* gene transcription [19]. Subjects with the less active long (L) allele (GT≥29) have been shown to exhibit an increased risk of oxidative stress-mediated conditions including cardiovascular diseases, as well as certain forms of cancer [20]. *UGT1A1* has also a highly

polymorphic promoter region with $(TA)_n$ repeat variations, modulating the *UGT1A1* transcriptional activity with clinically important consequences similar to those of the *HMOX1* promoter gene variants [20]. On the other hand, Bonkovsky *et al.* in their clinical study did not find any association of the variations of *HMOX1* gene promoter and responses to the first phase of antiviral therapy or to the likelihood of developing outcomes in HALT-C trial [21].

BLVR, the other enzyme involved in the heme catabolic pathway, is also implicated in the oxidative stress response [22]. Apart from its antioxidative effects, a cytoprotective action independent of heme degradation has been reported [23,24]. In fact, BLVR has been demonstrated to affect cell signaling pathways by regulating stress-responsive genes, including both *HMOX1* [25,26], and *HMOX2* [27]. Two isoforms of human BLVR, BLVRA (OMIM*109750) and BLVRB (OMIM*600941), products of different genes, have been described [28]. *BLVRA*, the major form of BLVR in the human adult liver, is subject to regulation by tumor necrosis factor-α, as well as by oxidative stress or hypoxia [29]. Importantly, biliverdin has been shown to inhibit HCV replication [30]. Lehmann *et al.* [30] recently demonstrated that biliverdin interferes with HCV replication-mediated oxidative stress by inducing the expression of antiviral interferons. Huang et al. [31] reported an association between sustained virological response (SVR) and expression of *BLVRB*, the embryonic form of BLVR in PBMC during the first weeks of antiviral therapy. However, no data are available on *BLVRA* expression in the liver, or PBL in chronic HCV infection.

2. Aims of the study

2.1. Study A. Gene expression analysis of *HMOX* and *BLVRA*

The study A was conducted to evaluate the possible role of HCV infection on *HMOX1/HMOX2/BLVRA* gene expression in the liver and PBL, as well as whether these genes may influence or predict the treatment response.

2.2. Study B. Genetic polymorphism analysis of *HMOX1* and *UGT1A1*

The study B was conducted to assess the effect of promoter variations of *HMOX1* and *UGT1A1* genes on the progression of fibrosis and treatment outcome in patients chronically infected with HCV.

3. Materials and methods

3.1. Studied groups

The study A was performed on 58 consecutive therapeutically naïve patients with chronic HCV infection. The patients were recruited between 2007 - 2011 at the Hepatology Center in the Central Military Hospital in Prague, Czech Republic. Patients with positivity of anti-HCV antibodies, and detectable HCV RNA in serum for at least 6 months, were included in the study. The patients received standard antiviral therapy (pegylated interferon alpha in combination with ribavirin (PEG-IFN-alpha/RBV)) according to EASL and AASLD practice guidelines [32]. The treatment regimens were: 1) PEG-IFN alpha 2a (Pegasys; Roche, Basel, Switzerland): 180 µg once weekly + RBV (Copegus; Roche, Basel, Switzerland) 1000 - 1200 mg daily, according to body weight (1000 mg ≤75 kg, 1200 mg > 75 kg); and 2) PEG-IFN alpha-2b (PegIntron; Schering-Plough, Kenilworth, NJ, USA): 1.5 µg/kg body weight once weekly + RBV (Rebetol; Schering-Plough, Kenilworth, NJ, USA): 1000 - 1200 mg daily, according to body weight (1000 mg ≤75 kg, 1200 mg > 75 kg). The total duration of the antiviral therapy was defined by the type of antiviral response within the first 12 - 24 weeks of

therapy. Patients with HCV RNA decrease < 2 log from baseline level at week 12 were classified as null responders and their antiviral therapy was terminated at week 12. Patients with HCV RNA decrease ≥ 2 log or with undetectable HCV RNA at week 12 were treated to week 24. If HCV RNA was detectable at week 24, patients were classified as slow responders and their therapy was terminated at week 24. Only patients with undetectable HCV RNA at week 24 were treated up to week 48. Based on the treatment response, patients were classified into two groups. Responders were defined as patients with sustained virologic response (SVR, undetectable HCV RNA at weeks 24, after completion of antiviral therapy, n=38). Treatment-failure patients (non-SVR, n=19) included those who did not achieved SVR (n=15) and patients who relapsed (n=5). For PBMC and PBL studies, 55 healthy volunteers (blood donors or employees of General Faculty Hospital and 1st Faculty of Medicine, Charles University in Prague) were used as control subjects.

The study B was performed on 146 patients diagnosed with chronic HCV infection. Chronic HCV infection was defined as the HCV RNA positivity in serum for at least 6 months and in whom other possible causes of liver disease were excluded (including HBV or HIV coinfections). A liver biopsy was performed in 112 patients by aspiration technique. Tissue samples were evaluated according to Ishak scoring system (33). In these patients, complete data on grading and staging of liver disease was available. A liver biopsy was refused by 19 patients; however, none of these patients showed clinical signs of liver cirrhosis. Clinically evident liver cirrhosis was found in 15 patients (signs of portal hypertension, hypersplenism or history of liver decompensation). Bioptically proven liver cirrhosis (stage 6) was detected in 10 patients, together forming the group of 25 liver cirrhosis patients. The control group consisted of 146 age- and sex-matched controls recruited from healthy blood donors. The study was registered under ID: NCT00842250 (www.clinicaltrial.gov). The study´s protocol

conformed to all ethical guidelines of the 1975 Declaration of Helsinki, as well as being approved by the Ethics Committee of the respective institutions. Additionally, all subjects in this study had provided written informed consent.

3.2. Sampling and storage

Patients' blood samples were collected on the day before treatment initiation (day 0, n=58), as well as at 12 (n=37), 24 (n=31), 36 (n=27) and 48 (n=16) weeks after initiation of the standard antiviral treatment. Samples for gene expression analyses were collected into PAXgene Blood RNA Tubes (PreAnalytix, Hombrechtikon, Switzerland) and stored at -80°C. Samples for determination of total HMOX activity in PBMC were collected in BD Vacutainer Blood collection tubes with heparin (BD Diagnostics-Preanalytical Systems, Franklin Lakes, NJ, USA). PBMC were isolated through a Ficoll-density gradient within 5 hours, and stored in potassium phosphate buffer at -80°C. The blood samples for analysis of interleukin 28B (*IL28B,* OMIM*607402*)* gene polymorphism were collected in BD Vacutainer Blood collection tubes with EDTA (BD Diagnostics-Preanalytical Systems, Franklin Lakes, NJ, USA) and stored at -80°C.

All liver samples were immediately placed into RNAlater (Ambion Diagnostics, Austin, TX, USA) and stored at -80°C until total RNA isolation.

3.3. HMOX activity in PBMC

Twenty µl of PBMC sonicate (2 million cells per reaction) were incubated for 15 min at 37°C in CO-free septum-sealed vials containing 20 µl of 150 µM methemalbumin (Sigma, St. Louis, MO, USA) and 20 µl of 4.5 mM NADPH (Sigma, St. Louis, MO, USA), as previously described [35]. Blank reaction vials contained 0.1 M phosphate buffer (Sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were of analytical grade,

and purchased from Penta (Prague, Czech Republic), pH=7.4 in place of NADPH. The reactions were terminated by adding 5 µl of 30% (w/v) sulfosalicylic acid (Sigma, St. Louis, MO, USA The amount of CO generated by the reaction and released into the vial headspace was quantified by gas chromatography (GC) with a Reduction Gas Analyzer (Trace Analytical Laboratories, now: AMETEK Process Instrument, Newark, DE, USA). HMOX activity was calculated as pmol CO/hr/10^6 cells.

3.4. RealTime HCV RNA and gene expression quantification

The liver tissue was homogenized using a MagNA Lyser System (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. Total RNA from the homogenized liver tissue was isolated using RNeasy Mini (Qiagen, Dallas, TX, USA), and total RNA from PBL using a PAXgene kit (Qiagen, Dallas, TX, USA), according to the manufacturer's instructions. DNase treatment with the RNase-free DNase (Qiagen, Dallas, TX, USA), prior to cDNA synthesis, was carried out according to the manufacturer's instructions. The RNA integrity was checked by agarose gel electrophoresis. First-strand cDNA was synthesized from 0,2 µg of total RNA in a final volume of 20 µl using a High-Capacity cDNA kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

The HCV Primer sequences were based on data by Carriere et al. [34]. Other primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/) and synthesized by Generi Biotech (Hradec Kralove, Czech Republic) (**Table 1**).

Table 1. Primer sequences for HCV RNA, target and internal control genes

	Forward primer	Reverse primer	Product PCR(bp)
HCV	GTCTAGCCATGGCGTTAGTA	CTCCCGGGGCACTCGCAAGC	246
HMOX1	GGGTGATAGAAGAGGCCAAGA	AGCTCCTGCAACTCCTCAAA	67
HMOX2	GAAGGAAGGGACCAAGGAAG	CTCCTCGAGGGCTGAGTATG	139
BLVRA	TCCCTCTTTGGGGAGCTTTC	GGACCCAGACTTGAAATGGAAG	180
HPRT	CACTGGCAAAACAATGCAGAC	GGGTCCTTTTCACCAGCAAG	92

HCV, hepatitis C virus; HMOX1, 2, heme oxygenase 1, 2; BLVRA, biliverdin reductase A; HPRT, hypoxanthine phosphoribosyltransferase.

To determine the relative expression level of all data analysis, HPRT expression levels were measured as internal controls. The delta cycle threshold value (Δct) was calculated from the given ct value by the formula: Δct= (ct sample – ct control). The fold change was calculated as ($=2^{-\Delta ct}$). Two reference genes (*HPRT, GAPD*) were selected as the most stable among 4 constant genes (*HPRT, GAPD, 18S RNA, UBC*) based on the analyses of 10 PBL of HCV infected patients and controls, and 10 liver samples of HCV infected patients by using geNORM 3.5 (http://medgen.ugent.be/genorm). Based upon similar expression levels as target genes, *HPRT* was found a more appropriate control gene, compared to *GAPD*.

qPCR was performed in 20 µl reaction volume, containing 4 µl of five-fold diluted cDNA template from completed RT reaction, 1x SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), and 200 nM forward and reverse primers. All RT-PCR were set up in 96-well optical plates, and run on an ABI PRISM 7500 Sequence Detector System (Applied Biosystems, Foster City, CA, USA).

The cycling conditions included polymerase activation at 95°C for 10 min, followed with 40 cycles of 95°C for 15 s, and 60°C for 60 s. PCR products were subjected to a melting curve analysis. All samples were analyzed in triplicates. Linearized constructs for the PCR validation procedure were prepared using a TOPO TA Cloning Kit, according to the

manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). PCR efficiencies for target and housekeeping cDNA were 97 - 105%.

3.5. DNA fragment analysis and IL28B genotyping

Genomic DNA was extracted from EDTA coagulated peripheral blood using MagNA Pure Compact Nucleic acid isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany). The $(GT)_n$ variations in *HMOX1* (dbSNP rs1805173) and $(TA)_n$ variations in the *UGT1A1* (dbSNP rs81753472) gene promoters were determined by fragment analysis as described previously (36). In brief, corresponding DNA fragments were amplified by duplex PCR, using following primers:

- HMOX1

forward: 5′ - ctgcagcttctcagatttcc - 3′
reverse: 5′ - acaaagtctggccataggac - 3′

- UGT1A1

forward: 5′ - gaacttggtgtatcgattggtttttgc - 3′
reverse: 5′ - catccactgggatcaacagtatcttcc - 3′

The reverse primers were labeled at the 5′ end with WellRED fluorescent dyes (Beckman Coulter, Fullerton, CA, USA). The resulting PCR products were separated on a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). Based on the number of $(GT)_n$ repeats, *HMOX1* alleles were classified into short S ($n < 24$), medium M ($n = 24$-28) and long L ($n \geq 29$) subgroups (37,38). The distribution of genotypes in control subjects was within Hardy-Weinberg equilibrium for both studied loci.

The human *IL28B* promoter polymorphism at position -3176 (rs12979860) was analysed using LightMix Kit IL28B (TIB Molbiol GmbH, Berlin, Germany).

3.6. Statistical analysis

The data are presented as the mean ± standard deviation (SD), or median (IQ range). Differences between the studied groups (HCV vs. controls, SVR vs. treatment-failure patients) were evaluated by an unpaired t-test, or Mann-Whitney U test. The significance of the relationship between *BLVRA* expression and the treatment outcome was determined by the chi-square test. Linear regression, Pearson's correlation and multivariate logistic regression analyses were performed using SigmaStat software, version 3.01, for the statistical analysis. For multiple logistic regression analysis following clinically relevant variables were included: *BLVRA* expression in PBL, *IL28B* gene variants, HCV RNA levels, stage of liver fibrosis, gender, hemoglobin levels and platelet number. The analyses comparing the frequency rates of different alleles as markers to phenotype parameters were based on the Fisher exact test.

All analyses were performed with alpha set to 0.05.

4. **Results**

4.1. Basal expression of BLVRA in PBL of HCV patients

BLVRA expression in PBL was markedly increased in HCV-infected patients before antiviral treatment, when compared to the control group (1.65±0.67 vs. 1.28±0.36, respectively, $p<0.001$). Simultaneously, baseline mRNA levels of *BLVRA* were significantly higher only in patients who achieved SVR, compared to the control group (1.82±0.74 vs. 1.28±0.36, respectively, $p=0.003$), but not in non-SVR patients (1.32±0.34 vs. 1.28±0.36 $p=0.65$). Significant differences in basal *BLVRA* expression were found between SVR and non-SVR patients (**Table 2**).

Table 2: Baseline characteristics of patients with hepatitis C

	Controls (N=55)	HCV (N=58)	SVR (N=38)	NVR (N=20)	*P*-value*
Gender (M:F ratio)	0.49	1.2	1.0	1.9	0.28
Age (years)	32.5 (27.8-44.0)	45.0 (38.0–53.8)	43.5 (37.3-51.8)	46.0 (43.5-54.0)	0.22
Viral load (log IU/ml)		6.05±0.67	6.05±0.75	6.05±0.50	0.98
Genotype HCV 1 (%)		84	76	94	0.20
IL28B(rs12979860)CC(%)		37	44	22	0.11
HMOX activity PBMC	36.3±18.1	20.6±16. 3 (N=53)	19.1±17.3 (N=34)	22.9±14.7 (N=19)	0.47
HMOX1 expression PBL	4.0±1.89	3.61±1.87	3.63±1.91	3.15±2.31	0.71
HMOX2 expression PBL	4.98±0.94	4.28±1.14	4.22±0.97	4.41±1.41	0.72
BLVRA expression PBL	1.28±0.36	1.68±0.68	1.82±0.74	1.32±0.34	0.003
Liver histology grading		5(4-5)	5(3-5)	4.5(4-6)	0.36
Liver histology staging		1(1-3)	1(1-2)	3.0(1-5)	0.03
Total bilirubin (µmol/l)		15.9 (12,4-19.4)	15.0 (11.4-20.6)	16.7 (14.6-18.3)	0.66
ALT (µkat/l)		0.97 (0.77-1.80)	0.93 (0.76-1.39)	1.08 (0.85-2.19)	0.95
AST (µkat/l)		0.67 (0.53-1.21)	0.63 (0.49-1.25)	0.79 (0.58-1.13)	0.71
ALP (µkat/l)		1.28 (0.99-1.95)	1.28 (1.05-2.02)	1.32 (0.99-1.83)	0.19
GGT (µkat/l)		0.68 (0.49-1.76)	0.57 (0.41-1.69)	1.15 (0.66-1.96)	0.73
Hemoglobin (g/l)		146.9±16.6	143.5±17	153.4±14.1	0.02
Platelets (x 10^9/l)		195.5±63.4	205.5±66.4	177.0±54.1	0.08

HCV, hepatitis C virus; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood leukocytes, SVR, responders; NVR, non-SVR patients; HMOX1, 2, heme oxygenase 1, 2; BLVRA, biliverdin reductase A; ALT, serum alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transpeptidase; N = number of patients. HMOX activity expressed as pmol CO/hr /10^6 cells. Data expressed as mean±standard deviation (SD), or median (IQ range). *P-value calculated between SVR and NVR.

When assessing possible factors responsible for treatment response, only *BLVRA* expression has been found to be a strong predictor (**Table 3**).

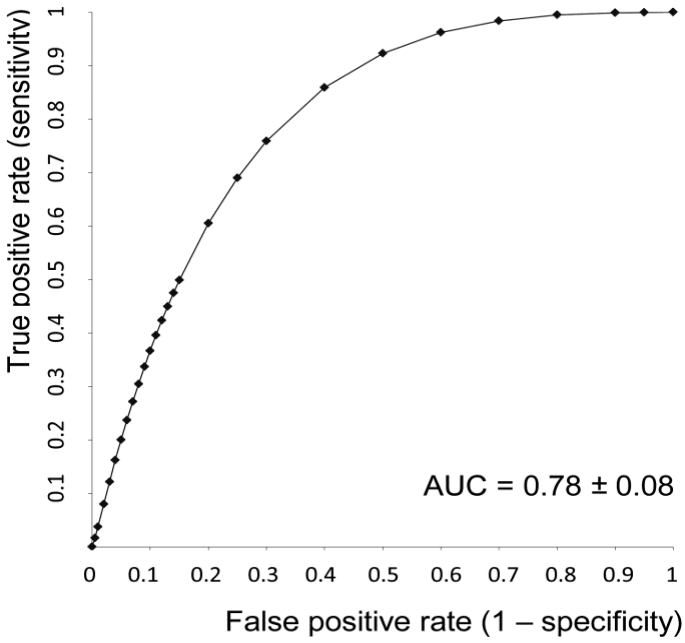
Table 3. Multivariate logistic regression analysis of potential SVR predictors

	OR	95% CI	P-VALUE
***BLVRA* expression PBL**	15	1.05 – 214.2	**0.046**
***IL28B* (rs12979860)**	3.05	0.39 – 24	0.29
HCV RNA	1.45	0.37 – 5.6	0.59
Liver fibrosis	0.76	0.4 – 1.5	0.4
Sex	1.81	0.13 – 2.5	0.66
Hemoglobin	0.94	0.88 – 1.01	0.09
Platelets	1.01	0.94 – 1,03	0.18

SVR, responders; HCV, hepatitis C virus; *IL28B*, interleukin 28B; PBL, peripheral blood leukocytes ; BLVRA, biliverdin reductase A; OR, odds ratio; CI, confidence interval.
IL28B genotype was tested as CC vs. non-CC allele carriers.

Exclusion of BLVRA from multivariant analysis did not have any effect on predictive value of tested variables. Based on ROC analysis (**Figure 2**), *BLVRA* expression predicted the treatment response with 76% sensitivity, 70% specificity (positive predictive value = 83%, negative predictive value = 61%).

Figure 2. ROC curve of *BLVRA* expression in peripheral blood leukocytes of HCV infected patients

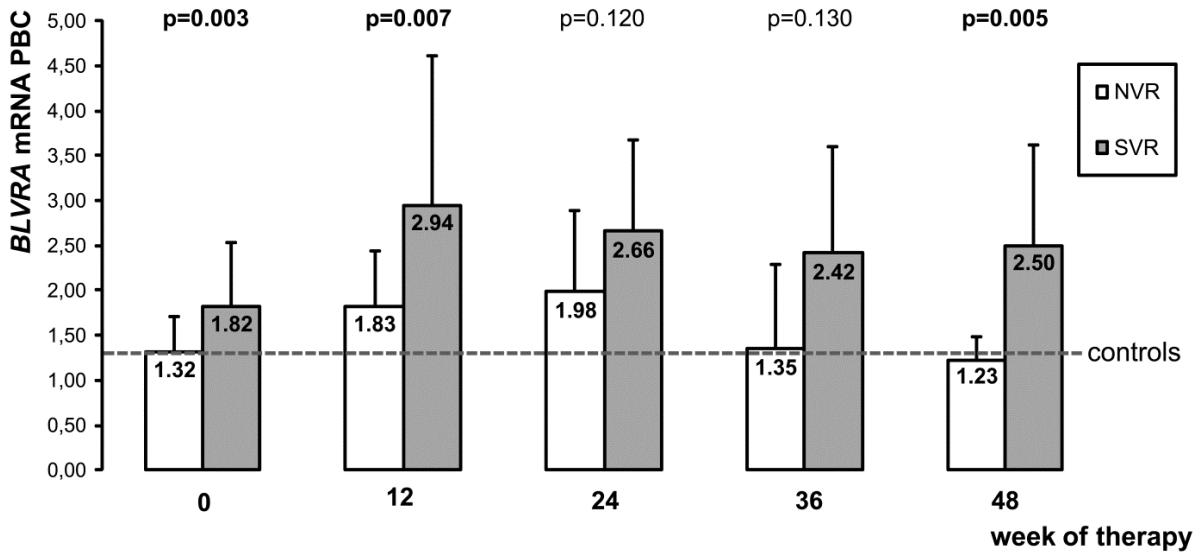


AUC, area under the ROC curve; ROC, Receiver Operating Charasteristic.

4.2. Expression of BLVRA in PBL in HCV patients during antiviral treatment

BLVRA expression significantly increased at weeks 12 (2.57±1.61, p=0.0004), 24 (2.57±1.17, p=0.0002), and 36 (2.16±1.19, p=0.03) after initiation of standard antiviral therapy when compared to the initial levels (1.68±0.68). Significant differences in *BLVRA* mRNA levels between SVR and non-SVR patients were found at weeks 12 and 48 after treatment initiation. Similar trends were also observed at weeks 24 and 36. These differences, however, did not reached statistical significance, most likely due to the low number of subjects (**Figure 3**).

Figure 3. *BLVRA* expression in peripheral blood leukocytes of responders and non-SVR patients during standard antiviral therapy



BLVRA expression was measured the day before treatment initiation (0), and 12, 24, 36 and 48 weeks after start of the standard treatment. Data represent means and standard deviations for triplicate determinations. P-values calculated between responders and non-SVR patients. BLVRA, biliverdin reductase A; PBL, peripheral blood leukocytes; SVR = responders, NVR = non-SVR patients.

Interestingly, *BLVRA* expression in SVR patients after withdrawal of antiviral therapy (n=10) decreased substantially compared to *BLVRA* expression levels at week 24 (median [IQ range] 1.38 [1.2-1.6] vs. 2.52 [1.5-2.8], p = 0.03), and reached control levels (median [IQ range] 1.38 [1.2-1.6] vs. 1.28 [1-1.5], p = 0.43).

4.3. *Expression and activity of* HMOX *in HCV patients*

Compared to controls, the HMOX activity in PBMC of HCV-infected patients before antiviral treatment was substantially reduced (20.6±16.3 vs 36.3±18.1 pmol $CO/10^6$ cells/h, respectively, p=0.001). Although PBL gene expression of *HMOX1* did not differ between HCV-infected patients and the control group, the *HMOX2* expression was slightly, but significantly, reduced in patients with HCV infection (4.28±1.14 vs 4.98±0.94, respectively,

p=0.001). While a significant correlation between PBL gene expression of *HMOX1*, *HMOX2* mRNA and HMOX activity in PBMC was only detected in control samples (r^2=0.210, p=0.007; r^2=0.113, p=0.03), this relationship was not observed in the HCV-infected patients (r^2=0.040, p=0.20; r^2=0.046, p=0.33). Finally, no differences in either HMOX activity or *HMOX* expression between SVR and non-SVR patients were detected.

4.4. *Correlation between BLVRA and HMOX mRNA levels in the liver and PBL*

No significant differences in pretreatment expression of *BLVRA* in the liver were found between SVR (n=18) and non-SVR patients (n=4) (0.35±0.24 vs. 0.34±0.24 p=0.97) most likely because of high variability of *BLVRA* expression in the liver compared to PBL. *BLVRA* expression, but not that of *HMOX1/HMOX2*, in the liver and PBL of HCV-infected patients were in direct relationship (n=13, r^2=0.347, p=0.03). No correlation was found between the mRNA levels of *HMOX1/HMOX2/BLVRA* and HCV RNA in the liver and PBL.

4.5. *HMOX promoter variants in HCV infected patiens*

The frequencies of S, M, and L alleles of the *HMOX1* gene promoter did not differ between HCV positive patients and control subjects (p>0.05). Analogously, no differences were found between cases and controls for the individual *HMOX1* genotypes (p>0.05).

No differences were also detected in the frequencies of the individual alleles between HCV patients without liver cirrhosis and those with liver cirrhosis (p>0.05 for all comparisons). This observation was confirmed in the detailed analysis of the possible role of *HMOX1* promoter gene variants on disease progression in HCV-infected patients who had undergone a liver biopsy. For this purpose, we grouped non-cirrhotic patients according to the staging into 3 subgroups: S 0-1 (n=43), S 2 (n=29) and more advanced patients S 3-4 (N=30). There were no significant associations between the frequency of individual *HMOX1* alleles

and the stage of the liver lesion ($p>0.05$ for all comparisons). As expected, patients with either a more advanced liver fibrosis, or cirrhosis, were significantly older than patients with lower stages ($p<0.005$).

As the S allele of the *HMOX1* gene seems to be associated with the lower incidence of oxidative stress-related diseases, we have analyzed in detail the frequency of this particular allele (genotypes SS/SL/SM) in the HCV infected patients with liver cirrhosis. Similarly, no difference was detected from this analysis ($p=1.00$).

4.6. UGT1A1 promoter variants in HCV-infected patients

In a comparable manner, non-significant findings were also detected for the *UGT1A1* promoter gene variants. The frequencies of alleles 6 and 7 of the *UGT1A1* gene promoter did not differ between HCV positive patients and healthy controls ($p>0.05$). No differences in the frequencies of alleles 6 and 7 of the *UGT1A1* gene were found between cirrhotic and non-cirrhotic HCV patients ($p=0.53$). As the *UGT1A1* 7/7 genotype has been reported to be associated with a lower incidence of oxidative stress-related diseases, we have analyzed the occurrence of this particular genotype in patients with or without liver cirrhosis compared to genotypes 6/7 and 6/6. No significant differences ($p=0.57$) were found.

4.7. HMOX1 and UGT1A1 promoter variants and HCV RNA viral load

In the final analysis, we focused on the possible protective role of both genetic variants on HCV RNA viral load. The baseline viral load was considered low if HCV RNA level at baseline (immediately before treatment initiation) was <800 000 IU/ml. Nevertheless, no significant difference in viral load was found in relationship to the presence of the *HMOX1* allele S or the *UGT1A1* 7/7 genotype.

5. **Discussion**

5.1. Study A. Gene expression analysis of *HMOX* and *BLVRA*

The precise molecular mechanisms underlying the responsiveness to antiviral treatment among HCV-infected individuals have yet to be completely identified. Because of the side effects and high costs of current antiviral therapy, it is very important to identify those markers that can discriminate among those patients who will respond to the standard treatment. Enzymes of the heme catabolic pathway seem to belong to such promising markers. The presence of short allele of the highly polymorphic *HMOX1* gene promoter was reported to be associated with higher HMOX1 inducibility leading, to increased cytoprotection. Indeed, higher basal *HMOX1* expression, as well as stronger inducibility in human endothelial cells carrying S allele, exposed to oxidative and inflammatory stimuli, has recently been described [21].

Based on these data, we have hypothesized, that the genetic variant in the *HMOX1* gene promoter might affect the response to chronic HCV infection as well as the progression of liver disease in chronically HCV infected patients. Our data, however, do not support this hypothesis. We were not able to prove any hepatoprotective effect of the S allele *HMOX1* carrier status, either on the HCV infection risk or on liver disease progression.

Similar findings were also obtained for *UGT1A1*. In contrast to other oxidative stress-mediated diseases, such as atherosclerosis, no associations were found for major *UGT1A1* gene promoter variations; either in HCV infection risk or liver disease progression. In addition, we were not able to find any association of these biologically relevant variants both of *UGT1A1*, as well as the *HMOX1* genes and hepatitis C viral load.

5.2. Study B. Genetic polymorphism analysis of *HMOX1* and *UGT1A1*

Zhu and coworkers [39] recently provided a plausible mechanism for the antiviral activity of HMOX1, demonstrating that the direct product of its activity, biliverdin, potently inhibits viral replication at biologically relevant concentrations in human hepatoma Huh-7.5 cells replicating HCV RNA, most likely *via* inhibition of HCV NS3/4A protease. In the current study, we prospectively investigated HMOX activity, as well as *HMOX1* expression in HCV-infected patients. Surprisingly, no difference in mRNA expression of *HMOX1* in PBL was found between therapeutically naïve HCV patients and controls, although the total HMOX activity in PBMC was significantly decreased in HCV patients before treatment, compared to the control group.

Furthermore, a correlation between the expression of *HMOX1*, *HMOX2*, and total HMOX activity was only detected in the control samples; not in the HCV-infected patients. In fact, interference of HCV with HMOX1 induction [40], reduced hepatic expression of *HMOX1* both *in vitro* and *in vivo* in HCV infection [8]; additionally, induced hepatic *HMOX1* expression *in vitro* were reported [17]. We hypothesized that *HMOX* and *BLVRA* gene expression in PBL can reflect their expression in the liver. In our study, due to unavailability of liver specimens of control subjects, correlation between the liver and PBL could be analyzed only in HCV patients. No association of *HMOX1/HMOX2* expression was found between the liver and PBL, and *HMOX1/HMOX2* and HCV RNA in the liver and PBL. On the other hand, expression of *BLVRA* in the liver tissue correlated with expression of *BLVRA* in PBL. The expression of *BLVRA* in PBL was higher in HCV-infected patients before antiviral therapy, compared to the control group; and subsequently increased 12, 24, and 36 weeks after initiation of standard antiviral therapy, when compared to initial levels. Most importantly, *BLVRA* expression in PBL was found to be strongly associated with response to the antiviral treatment. Recent genome-wide association studies identified strong evidence

IL28B gene variation (rs12979860) with SVR rates in patients chronically infected with genotype 1 HCV [41-43]. Although not statistically significant, a similar trend was observed in our cohort of patients for all genotypes (p=0.11) as well as for genotype 1 (p=0.16). The prevalence of CC vs non CC genotypes in our group of patients corresponds to prevalence rates of chronically infected HCV patients in the Czech Republic reported recently [44]. However, it should be noted that the lack of association with other clinically important variables tested in our regression model including *IL28B* gene variation might be due to small sample size effect. Furthermore, there was a clear trend for association of upregulated baseline *BLVRA* expression in PBL of patients with favorable CC genotype as compared to both non CC (p=0.059) and TT *IL28B* (p=0.058) patients.

Induced *BLVRA* gene transcription in PBMC of uninfected chimpanzees in response to IFN-alpha [45] and in INF-α treated PBMC [46] were previously reported, In accord with this data, *BLVRA* overexpression in our HCV-infected patients prior to and during antiviral treatment seems to be due to *BVLRA* upregulation by INF-alpha [45, 46]. Moreover, our results showing an association of *BLVRA* in PBL with the treatment outcome are in agreement with substantially greater global induction of IFN-stimulated genes observed in the PBMC of treatment responders [47].

This data are in accord with our observation indicating that 1) SVR patients have increased *BLVRA* expression prior initiation of therapy (likely due to endogenous interferon induced by HCV infection); 2) both SVR and NVR patients have increased *BLVRA* expression during antiviral therapy (likely due to exogenous interferon administered therapeutically); 3) SVR patients after withdrawal of antiviral therapy have decreased expression of *BLVRA* to control values (likely due to decreased production of endogenous interferon, since HCV, as the major stimulus, is absent). It is also important to note, that *BLVRA* expression during HCV infection and antiviral therapy is independent of ribavirin-induced hemolysis. However, our data does

not provide conclusive evidence whether *BLVRA* expression is involved actively in driving the treatment response, or is just a surrogate marker for treatment responsiveness.

In conclusion, our pilot results demonstrate that patients with chronic HCV infection significantly upregulate *BLVRA* expression in PBL, closely correlating with those in liver tissue. In addition, basal *BLVRA* expression in PBL is strongly associated with response to treatment. Finally, the lack of *BLVRA* overexpression is associated with non-responsiveness to standard antiviral therapy. Nevertheless, larger prospective studies are needed to confirm our data.

6. Conclusions

6.1. Study A. Gene expression analysis of *HMOX* and *BLVRA*

We have established the new sensitive method for analysis of HMOX activity in PBMC – based on reduction gas chromatogramy and developed RealTime PCR assay for *HMOX1/2* and *BLVRA* expression in the liver and peripheral blood leukocytes (PBL). HMOX activity in PBMC and *HMOX* expression in PBL of HCV infected patients did not have any effect on the treatment outcome. Compared to the controls, substantially increased *BLVRA* expression was detected in PBL of therapeutically naïve HCV patients. A marked difference in *BLVRA* expression in PBL between the sustained responders and patients with treatment failure was detected at week 0 and during the follow-up. Correlation analysis of *BLVRA* expression showed significant positive relationship between the liver and PBL in HCV infected patients. Multivariate analysis revealed that *BLVRA* basal expression in PBL was an independent predictor for sustained virological response.

6.2. Study B. Genetic polymorphism analysis of *HMOX1* and *UGT1A1*

We have determined the (GT)n and (TA)n dinucleotide variations in *HMOX1* and *UGT1A1* gene promoters by fragment analysis in patients with chronic HCV infection and age- and sex-matched healthy subjects. No differences were found in the frequencies of each particular allele of both genes, between HCV patients and a control group. Furthermore, no association was detected between either the *HMOX1* or the *UGT1A1* promoter variants and the individual histological stages of liver disease in the HCV positive patients. Based on our data, microsatellite variations in the *HMOX1* and *UGT1A1* genes are not likely to protect from progression of liver disease in patients with chronic HCV infection.

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8. List of original articles

1. Publications *in extenso* related to the thesis

a) with IF

Subhanova I, Muchova L, Lenicek M, Vreman HJ, Luksan O, Kubickova K, Kreidlova M, Zima T, Vitek L, Urbanek P. Expression of biliverdin reductase A in peripheral blood leukocytes is associated with treatment response in HCV-infected patients.

PLOS ONE 2013; 8 (3) e57555. IF 4,09

Urbanek P, Lenicek M, Muchova L, Subhanova I, Dusek L, Kasprikova N, Hrabal P, Bruha R, Vitek L: No association of promoter variations in *HMOX1* and *UGT1A1* genes with liver injury in chronic hepatitis C.

Annals of Hepatology 2011;10(4): 445-451. IF 1,81

b) without IF

Urbánek P, Subhanová I, Janoušová E, Dušek L, Mareček Z, Brůha R, Petrtýl J, Brodanová M: Účinnost terapie pegylovaným interferonem a ribavirinem u pacientů s chronickou HCV infekcí.

Vnitřní lékařství 2009; 55(5):474-479.

Friedecký B, Novotný D, Pavlík E, Plíšková L, Riedlová P, Subhanová I, Zima T, Žůrek D: Validace a verifikace molekulárně biologických metod založených na analýze extrahumánního genomu.

Klinická biochemie a metabolismus 2006;2: 118-124.

2. Publications *in extenso* with different objectives

a) with IF

Šperl J, Prochazkova J, Martásek P, Subhanová I, Fraňková S, Trunečka P, Jirsa M: N-acetyl Cysteine Averted Liver Transplantation in a Patient with Liver Failur Caused by Erytropoietic Protoporphyria.

Liver Transplantation 2009; 15(3): 352-354. **IF 3,4**

Šperl J, Petrášek J, Fraňková S, Farrag S. M, Subhanová I, Vítek L, Jirsa M, Špičák J, Martásek P: Improvement of Liver Dysfunction after Treatment with N-acetyl cysteine in Patient with Erythropoetic Protoporphyria.

Journal of Hepatology 2008; 48: S333. **IF 6,6**

Hřebíček M, Jirásek T, Hartmannová H, Nosková L, Stránecký V, Ivánek R, Kmoch S, Cebecauerová D, Vítek L, Mikulecký M, Subhanová I, Hozák P, Jirsa M: Rotor - type hyperbilirubinaemia has no defect in the canalicular bilirubin export pump.

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Cebecauerova D, Jirásek T, Budisová L, Mandys V, Volf V, Novotná Z, Subhanová I, Hřebíček M, Elleder M, Jirsa M: Dual Hereditary Jaundice: Simultaneous Occurence of Mutations Causing Gilbert´s and Dubin-Johnson Syndrome.

Gastroenterology 2005; 129(1): 315-320. **IF 13,0**