ORIGINAL ARTICLE

Relationship between unconjugated hyperbilirubinemia and lipoprotein spectrum

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Abstract

OBJECTIVES: The aim of this study was to find out the relationship between unconjugated hyper-bilirubinemia and the occurrence of atherogenic plasma lipoproteins with the emphasis on the small dense LDL particles, in the individuals with Gilbert's syndrome. We used a new electrophoretic method, which enables to analyze up to 12 lipoprotein subfractions. Atherogenic lipoprotein profile is characterized by the presence of very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and the presence of small dense LDL lipoproteins. The presence of LDL1 and LDL2 subfractions, as well as HDL lipoproteins is considered as a protective factor.

METHODS: Molecular-genetic examination of Gilbert's syndrome using fragment analysis method was carried out in collaboration with the Centre for Medical Genetics, University Hospital in Bratislava. Total cholesterol and triglycerides in plasma were analyzed from lipid parameters by means of enzymatic CHOD-PAP method, Roche Diagnostics, Germany. Biochemical parameters – bilirubin (total, conjugated and unconjugated), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GMT), (Roche Diagnostics, Germany), TSH, FT3, fT4 (Siemens) were also examined. Serum lipoproteins and their subfractions were examined using Lipoprint LDL System Quantimetrix, CA, USA (12).

RESULTS: We found significantly higher levels of total bilirubin and unconjugated bilirubin in patients with Gilbert's syndrome. In the control group, probands had significantly higher triglycerides levels, VLDL cholesterol levels, IDL cholesterol level, and small dense LDL levels compared to the group with Gilbert's syndrome. Probands with Gilbert's syndrome had significantly lower presence of atherogenic lipoprotein spectrum than probands in control group (5% vs. 18%). We found significantly negative correlation between serum unconjugated bilirubin levels and LDL 3–7 ($r = -0.594$, $p<0.01$), as well as between bilirubin and triglycerides ($r = -0.540$, $p<0.01$). Serum bilirubin concentration and LDL 1–2 concentration correlated significantly positively ($r = 0.451$, $p<0.05$).

CONCLUSION: The presence of an atherogenic lipoprotein spectrum is determined by the particular representation of small dense LDL. Atherogenic spectrum was significantly lower in patients with Gilbert's syndrome compared to the control (5% vs. 18%). In our study, we did not follow the risk of coronary heart disease or other manifestations of atherosclerotic arteries disability. However, we found the inverse

relationship of serum bilirubin levels and atherogenic small dense LDL. We found out that the protective antiatherogenic effect of hyperbilirubinemia is potentiated by low occurence of strongly atherogenic small dense LDL and persons with hyperbilirubinemia (in our case represented Gilbert's syndrome), could be protected against the development of atherosclerosis.

Abbreviaions and units:

ALT	- alanine aminotransferase
ALP	- alkaline phosphatase
AST	- aspartate aminotransferase
BMI	- body mass index
CHD	- coronary heart disease
GMT	- gamma glutamyl transpeptidase
GS	- Gilbert's syndrome
IDL	- intermediate density lipoproteins
VLDL	- very low density lipoproteins
HDL	- High density lipoproteins
LDL	- Low density lipoproteins
TA	- Tymidin-adenine
UGT1A1	- UDP-glucuronyltransferase
UGT1A1*28	- Localisation of the gene for UGT1A1 on the allele 28

INTRODUCTION

Atherosclerosis is the leading cause of morbidity and mortality in developed countries. There are many mechanisms involved in the pathogenesis of atherosclerosis. Among them LDL (low density lipoproteins) and other lipoproteins play an important role. In 1989, Steinberg and co-workers presented the hypothesis of oxidative modification of LDL in atherogenesis (Steinberg et al. 1989), which was later completed (replenished) by Stocker and Keaney (Stocker & Keaney 2004). Oxidized LDL can stimulate foam cell formation, muscle cell apoptosis, enhance release of proinflammatory cytokines and easier penetrate into the subendothelial space (Oravec *et al.* 2011). If oxidation of LDL is an essential part of atherosclerosis, then its inhibition should limit it. Long-term epidemiological and clinical studies suggest that increased intake of certain antioxidants, e.g. vitamins, microelements and other biologically active compounds can reduce the risk of cardiovascular diseases, as well as cancer and other diseases.

In opposite to exogenous antioxidants, we have considerably less information about endogenous antioxidants and their mechanism of protection. Among them bilirubin is considered as a potent protective factor for tissues. It exhibits antioxidant, antimutagenic and anticomplement activity and is considered one of the most important antioxidants *in vitro* and *in vivo* whether it is unconjugated, conjugated, free or bound to albumin. It can prevent oxidation of LDL lipoproteins, efficiently scavenge peroxyl radicals and therefore acts against atheroma formation and progression of atherosclerosis (Marilena 1997). Antiatherosclerotic properties of bilirubin have been confirmed in several *in vitro* studies, as well as in animal experiments.

In 1994, Schwertner *et al.* found that reduction of serum bilirubin levels on half the benchmarks led to a 47% increase in the likelihood of developing more severe forms of coronary heart disease (CHD) (Schwertner *et al.* 1994). Hopkins *et al.* confirmed inverse relationship of serum bilirubin concentration and cardiovascular disease in men and women. There was an 80% reduction in the risk of CHD in subjects with bilirubin levels at the upper limit of normal compared to those in the lower limit of reference interval. Similar association has also been found with other consequences of atherosclerosis such as peripheral arterial disease and stroke (Hopkins *et al.* 1996). Inverse relationship between serum bilirubin and risk of myocardial infarction, coronary heart disease and peripheral artery disease has also been confirmed in the Framingham Offspring Study which included 4276 people, men and women. Djousse *et al.* studied the dependence of serum bilirubin and any expression of coronary heart disease. Positive correlation was found mainly in men, while in women this relationship was not clearly proven (Djoussé *et al.* 2001). Meta-analysis of eleven studies confirmed that the serum bilirubin concentration is inversely related to the severity of atherosclerosis in men. Based on these studies, an increase in serum bilirubin level of 1 μmol/l led to a 6.5% reduction in CHD (Lin *et al.* 2006; Novotný & Vítek 2003).

The first study, which followed the risk of CHD in individuals with Gilbert's syndrome (GS), was performed by Vítek and co-workers. The subjects in this study had chronic unconjugated hyperbilirubinemia in the absence of liver disease and hemolysis. Approximately 2% of these individuals had CHD compared to 12.1% in other populations and significantly reduced risk was also observed for another 3 years of observation. Individuals with GS had also higher levels of HDL cholesterol and higher total serum antioxidant capacity than the control group. As a result we found that slightly elevated serum bilirubin level (33 ± 14 μmol/l) is associated with lower prevalence of coronary heart disease and lower risk of atherosclerosis development (Vítek *et al.* 2002; Schwertner & Vítek 2008).

The aim of this study was to analyze the serum bilirubin level, lipid and lipoprotein parameters with the emphasis on the presence of atherogenic small dense LDL in patients with Gilbert's syndrome, in order to confirm the multiplication of the protective effect of hyperbilirubinemia and low concentration of atherogenic lipoproteins in case that low concentration of these will be present.

PATIENTS AND METHODS

Patients

In our study 150 volunteers were examined of which 100 persons according to exclusion criteria were selected (Table 1).

Tab. 1. Exclusion criteria.

Exclusion criteria
- thyroid disease - liver disease - diagnosis of arterial hypertension, ischemic heart disease, diabetes mellitus - alcohol and other drug abuse - kidney disease - malabsorption syndrome

Tab. 2. Basic characteristics of groups.

	Group 1 n = 40	Group 2 n = 60
Age (mean ± SD)	29.7 ± 6.57	31.1 ± 9.06
Age range	19–47	17–62
Body mass index ± SD	24.5 ± 3.63	24.6 ± 2.52
Smokers	15 (37.5%)	32 (53.3%)
Non-smokers	25 (62.5%)	28 (46.7%)

Tab. 3. Summary table.

	Group 1 (Gilbert's syndrome)	Group 2 (control group)	Signif.
Number of probands	40	60	NS
Total bilirubin (μmol/l)	25.91 ± 12.80	9.73 ± 4.60	$p<0.001$
Conjugated bilirubin (μmol/l)	6.98 ± 1.44	8.84 ± 3.11	NS
Unconjugated bilirubin (μmol/l)	16.23 ± 10.05	4.89 ± 8.26	$p<0.001$
Total cholesterol (mmol/l)	4.49 ± 0.82	4.84 ± 1.19	NS
VLDL cholesterol (mmol/l)	0.51 ± 0.19	0.66 ± 0.24	$p<0.01$
IDL cholesterol (mmol/l)	0.93 ± 0.25	1.11 ± 0.40	$p<0.01$
LDL cholesterol (mmol/l)	2.66 ± 0.60	2.92 ± 0.98	NS
LDL 1-2 subfractions (mmol/l)	1.72 ± 0.47	1.74 ± 0.70	NS
LDL 3-7 subfractions (mmol/l)	0.010 ± 0.023	0.070 ± 0.187	$p<0.05$
HDL cholesterol (mmol/l)	1.32 ± 0.30	1.25 ± 0.31	NS
Triglycerides (mmol/l)	1.01 ± 0.47	1.23 ± 0.48	$p<0.05$
AST (μkat/l)	0.41 ± 0.14	0.39 ± 0.08	NS
ALT (μkat/l)	0.46 ± 0.27	0.40 ± 0.12	NS
GMT (μkat/l)	0.35 ± 0.19	0.42 ± 0.25	NS
ALP (μkat/l)	0.92 ± 0.26	0.99 ± 0.26	NS
TSH (mIU/l)	1.99 ± 0.86	2.23 ± 1.14	NS
fT_4 (pmol/l)	14.67 ± 2.91	15.51 ± 2.69	NS
fT_3 (pmol/l)	5.09 ± 0.77	5.01 ± 0.75	NS

AST – aspartate aminotransferase, ALT – alanin aminotransferase, GMT – gama glutamyl transpeptidase, ALP – alkaline phosphatase, TSH – thyroid stimulating hormone, fT4 – free T4, fT3 – free T3

Based on the molecular-genetic testing for the presence of mutation in the promoter gene for bilirubin-UDP-glucuronosyltransferase (UGT1A1) respondents were divided in two groups. The group 1 consisted of 40 probands confirmed to have Gilbert's syndrome. In the group 2 - control group there were 60 subjects in whom the diagnosis of Gilbert's syndrome was excluded. The mean age in the group 1 was 29.7 years (19 to 47 years) and in the group 2 was 31.1 years (17 to 62 years). Basic characteristics of both groups are shown in Table 2.

Methods

Respondents completed a short questionnaire. The questionnaire consisted of name, age, sex, questions focused on the presence of thyroid disease, hypertension, diabetes mellitus, malabsorption, renal diseases, drug history, questions about the abuse of nicotine, drugs. The anthropometric parameters of weight and height were measured and body mass index (BMI) was determined.

Molecular-genetic examination of Gilbert's syndrome using fragment analysis method was carried out in collaboration with the Centre for Medical Genetics, University Hospital in Bratislava. Gilbert's syndrome (GS) occurs in approximately 10% of the European population. The most common cause is homozygosity for UGT1A1*28, which is a TA repeated expression in the promoter of UGT1A1 (Bosma *et al.* 1995).

Total cholesterol and triglycerides in plasma were analyzed from lipid parameters by means of enzymatic CHOD-PAP method, Roche Diagnostics, Germany. Biochemical parameters – bilirubin (total, conjugated and unconjugated), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GMT), (Roche Diagnostics, Germany), TSH, FT3, fT4 (Siemens) were also examined.

Serum lipoproteins and their subfractions were examined using Lipoprint LDL System Quantimetrix, CA, USA (Hoefner *et al.* 2001). With this method twelve lipoprotein classes and their subfractions can be identified: very low density lipoproteins (VLDL), three subfractions of intermediate density lipoproteins (IDL 1–3), seven subfractions of low density lipoprotein (LDL 3–7) and high density lipoproteins (HDL). Atherogenic lipoprotein profile is characterized by a predominant presence of atherogenic lipoproteins: VLDL, IDL and small dense LDL. The latter ones, represent highly atherogenic LDL subfractions forming fractions LDL 3–7. Counterweight to atherogenic lipoproteins are non-atherogenic lipoproteins: HDL, LDL1 and LDL2. Non-atherogenic lipoprotein phenotype A and an atherogenic lipoprotein phenotype B were also determined by Lipoprint LDL System.

The blood samples were taken from the cubital vein after 12 hour of fasting. EDTA-K2 plasma was obtained and used for analyzing the lipid and biochemical parameters.

Statistical analysis was performed with the use of descriptive statistics, Pearson and Spearman correlation, student's t-test for unpaired observations. Values of $p<0.05$ were accepted as statistical significant.

RESULTS

In the group 1 we found significantly higher levels of total bilirubin and unconjugated bilirubin. In the control group, probands had significantly higher triglycerides levels, VLDL cholesterol levels, IDL cholesterol level, and small dense LDL levels compared to the group with Gilbert's syndrome (Table 3). Probands in the group 1 had significantly lower occurence of atherogenic lipoprotein spectrum (Table 4).

Analysis of bilirubin, lipid and lipoprotein parameters

Correlation analysis between serum bilirubin levels, lipids and lipoprotein parameters was done separately for group 1 and 2 (Tables 5 and 6).

In the group 1 we found significantly negative correlation between serum unconjugated bilirubin levels and LDL 3–7 (r = –0.594, $p<0.01$), as well as between bilirubin and triglycerides (r = –540, $p<0.01$). Serum bilirubin concentration and LDL 1–2 concentration correlated significantly positively (r = 0.451, $p<0.05$). In the group 2 there was significant negative correlation between serum bilirubin levels and LDL 3–7 (r = –0.652, $p<0.05$), too.

Tab. 4. Occurrence of atherogenic vs. non-atherogenic lipoprotein phenotype (phenotype B vs. phenotype A).

	Group 1 (n=40)	Group 2 (n=60)	Signif.
Non-atherogenic profile	38 (95%)	49 (82%)	NS
Atherogenic profile	2 (5%)	11 (18%)	$p<0.05$

DISCUSSION

The aim of our study was to analyze the lipoprotein spectrum in relation to serum levels of unconjugated bilirubin. We used a new electrophoretic method, which enables to analyze up to 12 lipoprotein subfractions. Atherogenic lipoprotein profile is characterized by the presence of very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL) and the presence of small dense LDL lipoproteins. The presence of LDL1 and LDL2 subfractions, as well as HDL lipoproteins is considered as a protective factor.

We found that subject with Gilbert's syndrome had significantly lower serum levels of small dense LDL, VLDL and triglycerides. Our results are consistent with observations of the authors Schwertner and Hopkins (Schwertner *et al.* 1994; Hopkins *et al.* 1996; Schwertner & Vítek 2008). They observed a negative correlation between bilirubin and LDL cholesterol. In

Tab. 5. Correlation analysis in group 1 – Gilbert's syndrome.

Group 1	Bi (µmol/l)	Chol (mmol/l)	VLDL (mmol/l)	IDL (mmol/l)	LDL (mmol/l)	LDL1,2 (mmol/l)	LDL 3-7 (mmol/l)	HDL (mmol/l)	TAG (mmol/l)
Bi (µmol/l)	–	–0.265	–0.349	0.019	–0.348	0.451*	–0.594**	0.218	–0.540**
LDL 3-7 (mmol/l)	–0.594**	–0.104	0.137	–0.363*	–0.026	0.107	–	–0.322*	0.486**
TAG (mmol/l)	–0.540**	0.294	0.687**	0.1	0.317*	0.326*	0.486**	–0.27	–

*$p<0.05$; **$p<0.01$
Bi – bilirubin, Chol – cholesterol, IDL – intermediate density lipoproteins, LDL – low density lipoproteins, HDL – high density lipoproteins, TAG - triglycerides

Tab. 6. Correlation analysis in group 2 – control group.

Group 1	Bi (µmol/l)	Chol (mmol/l)	VLDL (mmol/l)	IDL (mmol/l)	LDL (mmol/l)	LDL1,2 (mmol/l)	LDL 3-7 (mmol/l)	HDL (mmol/l)	TAG (mmol/l)
Bi (µmol/l)	X	–0.284	–0.414	0.563	0.341	0.234	–0.652*	0.223	–0.341
LDL 3-7 (mmol/l)	–0.652*	0.158	0.170	–0.095	0.162	0.014	X	–0.018	0.038
TAG (mmol/l)	–0.341	0.426**	0.701**	0.441**	0.345**	0.237	0.038	–0.061	X

*$p<0.05$; **$p<0.01$

our study, persons with Gilbert's syndrome had significantly lower presence of atherogenic lipoprotein spectrum. This showed that the anti-atherogenic effect of hyperbilirubinemia in persons with GS is potentiated by a presence of non-atherogenic lipoproteins. We also confirmed a positive correlation between serum bilirubin levels and HDL cholesterol. This further underlines anti-atherogenic properties in plasma in persons with Gilbert's syndrome. Other published observations of Schwertner and Hopkins brought brought an evidence, that a decrease in serum bilirubin on a half the benchmarks, led to a 47% increase in the likelihood of developing more severe forms of coronary heart disease. These results are consistent with our findings. Vítek with co-workers also published similar results (Vítek et al. 2002).

Yet published scientific works, which were focused on the assessment of the protective properties of bilirubin and its relation to lipid parameters did not work with techniques that quantify small dense lipoproteins. In the analysis of subfractions of LDL in our study we observed a significant positive correlation between serum bilirubin levels and small dense LDL in both groups. Respondents with Gilbert's syndrome had higher levels of unconjugated bilirubin and lower triglycerides. Higher bilirubin concentrations were accompanied with higher protective LDL 1–2 levels in a group of persons with Gilbert's syndrome.

The presence of atherogenic lipoprotein spectrum is determined by the particular representation of small dense LDL. Atherogenic spectrum was presented significantly less in patients with Gilbert's syndrome compared to the control group (5% vs. 18%).

In our study, we have not followed the risk of coronary heart disease or other manifestations of atherosclerotic arteries disability. However, we found the inverse relationship of serum bilirubin levels and atherogenic small dense LDL levels. If one of the conditions of atherogenesis is the presence of small dense LDL, we can say that healthy persons with hyperbilirubinemia (in our case represented Gilbert's syndrome), could be protected against atherosclerosis. Further studies are needed to find out the exact mechanism of protective effect of hyperbilirubinemia in Gilbert's Syndrome.

REFERENCES

1 Bosma PJ, Chowhudry JR, Bakker C, *et al.* (1995) The genetic basis of the reduced expression of bilirubin UDP-glucuronyltransferase 1 in Gilbert's syndrome. N Engl J Med. **333**(18): 1171–5.
2 Djoussé L, Levy D, Cupples LA, *et al.* (2001) Total serum bilirubin and risk of cardiovascular disease in the Framingham offspring study. Am J Cardiol. **87**(10): 1196–1200; A4,7.
3 Hoefner DM, Hodel SD, O´Brien JF, *et al.* (2001) Development of a Rapid, Quantitative Method for LDL subfraction with Use of the Quantimetrix Lipoprint LDL System. Clin Chem. **47**(2): 266–274.
4 Hopkins PN, Wu LL, Hunt SC, *et al.* (1996) Higher Serum Bilirubin Is Associated With Decreased Risk for Early Familial Coronary Artery Disease. Arterioscler Thromb Vasc Biol. **16**(2): 250–255.
5 Lin JP, O'Donnell ChJ, Schwaiger JP, *et al.* (2006) Association Between the UGT1A1*28 Allele, Bilirubin Levels, and Coronary Heart Disease in the Framingham Heart Study. Circulation. **114**(14): 1476–1481.
6 Marilena G (1997) New Physiological Importance of Two Classic Residual Products: Carbon Monoxide and Bilirubin. Biochem Mol Med. **61**(2): 136–142.
7 Novotný L, Vítek L (2003) Inverse Relationship Between Serum Bilirubin and Atherosclerosis in Men: A Meta-Analysis of Published Studies. Exp Biol Med (Maywood). **228**(5): 568–571.
8 Oravec S, Dukat A, Gavornik P, Caprnda M, Kucera M, Ocadlik I (2011) Contribution of the atherogenic lipoprotein profile to the development of arterial hypertension. Bratisl Lek Listy. **112**(1): 4–7.
9 Schwertner HA, Jackson WG, Tolan G (1994) Association of low serum concentration of bilirubin with increased risk of coronary artery disease. Clin Chem. **40**(1): 18–23.
10 Schwertner HA, Vítek L (2008) Gilbert syndrome, UGT1A1*28 allele, and cardiovascular disease risk: Possible protective effects and therapeutic applications of bilirubin. Atherosclerosis. **198**(1): 1–11.
11 Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL (1989) Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med. **320**(14): 915-924.
12 Stocker R, Keaney JF Jr. (2004) Role of Oxidative Modifications in Atherosclerosis. Physiol Rev. **84**(4): 1381–1478.
13 Vítek L, Jirsa M Jr, Brodanová M, *et al.* (2002) Gilbert syndrome and ischemic heart disease: a protective effect of elevated bilirubin levels. Atherosclerosis. **160**(2): 449–456.