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REVIEW

Bilirubin and Atherosclerotic Diseases

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Summary

Bilirubin is the final product of heme catabolism in the systemic circulation. For decades, increased serum/plasma bilirubin levels were considered an ominous sign of an underlying liver disease. However, data from recent years convincingly suggest that mildly elevated bilirubin concentrations are associated with protection against various oxidative stress-mediated diseases, atherosclerotic conditions being the most clinically relevant. Although scarce data on beneficial effects of bilirubin had been published also in the past, it took until 1994 when the first clinical study demonstrated an increased risk of coronary heart disease in subjects with low serum bilirubin levels, and bilirubin was found to be a risk factor for atherosclerotic diseases independent of standard risk factors. Consistent with these results, we proved in our own studies, that subjects with mild elevation of serum levels of unconjugated bilirubin (benign hyperbilirubinemia, Gilbert syndrome) have much lower prevalence/incidence of coronary heart as well as peripheral vascular disease. We have also demonstrated that this association is even more general, with serum bilirubin being a biomarker of numerous other diseases, often associated with increased risk of atherosclerosis. In addition, very recent data have demonstrated biological pathways modulated by bilirubin, which are responsible for observed strong clinical associations.

Key words

Bilirubin • Atherosclerosis • Benign hyperbilirubinemia • Cardiovascular diseases • Gilbert syndrome

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Introduction

Serum bilirubin, for decades considered an ominous sign of an underlying liver disease and unfavorable prognostic factor, seems to affect biological processes in much wider consequences. Bilirubin, formed primarily from senescent red blood cells *via* the heme catabolic pathway in the reticulo-endothelial system has several unique biological properties. Due to a system of conjugated double bonds within its molecule, it is a potent endogenous antioxidant (Stocker *et al.* 1987). It is also a non-competitive inhibitor of oxidative as well as protein phosphorylation (Hansen *et al.* 1996) with widespread biological effects, including energy homeostasis, enzyme and membrane functions, protein synthesis, RNA/DNA metabolism, and immunologic mechanisms (Hansen *et al.* 1996). Quite recently, bilirubin was demonstrated to be a natural PPARα ligand (Stec *et al*. 2016) further enlarging its potential to affect metabolic functions in a human body (Hinds *et al.* 2016).

This review follows our previous review papers on this topic (Lin *et al.* 2010, Schwertner and Vitek 2008, Vitek 2012, Vitek and Ostrow 2009, Vitek and Schwertner 2007, Wagner *et al.* 2015) with the main focus on the contribution of our group to the clinical research of bilirubin and atherosclerosis, demonstrating also further perspectives of this interesting molecule.

Bilirubin as a potent endogenous antioxidant

However, the whole story started much earlier than in 90´s. A potential physiological role for bilirubin was proposed as early as 1954 by Bernhard *et al.*, who had shown that small quantities of bilirubin had been able

to prevent the oxidation of vitamin A and unsaturated fatty acids. Similar results on the inhibition polyunsaturated fatty acid oxidation by bilirubin were published decades later by Japanese investigators (Onishi *et al*. 1971) as well as in a landmark study by Stocker *et al.* (1987). Also additional studies confirmed huge antioxidant potential of bilirubin, such as that of Wu *et al.* (1994) who demonstrated that bilirubin is 20-times more effective in preventing LDL oxidation than Trolox, a vitamin E analogue. In addition, it was demonstrated in another study that 10 nmol/l of bilirubin, exerting its antioxidant activities in so-called biliverdin-bilirubin redox cycle, protects against almost 10,000-fold higher concentrations of H_2O_2 (Baranano *et al.* 2002).

Bilirubin as an anti-atherogenic molecule – lessons from the clinical studies on cardiovascular diseases

In 1994, Schwertner *et al.* were the first researchers who reported in their large epidemiological study on almost 900 males a negative association between fasting serum bilirubin concentrations and ischemic heart disease (IHD). The strength of this association was similar to that of smoking, systolic blood pressure, and HDL-cholesterol. Early after publication of this report, we have initiated a retrospective study on subjects with Gilbert syndrome (also known as benign hyperbilirubinemia, characterized with mild elevation of unconjugated bilirubin in the absence of any underlying liver disease and/or hemolysis) (Vitek *et al.* 2002). Although the cohort was small (n=50), the individuals with benign hyperbilirubinemia had a prevalence rate for IHD of 2 % compared to 12.1 % for the general population. The study was switched to prospective fashion and subjects were followed for the next 3 years. During this period of follow-up virtually no case of IHD was diagnosed in the population with Gilbert syndrome as compared to significantly higher predicted 3-year incidence of IHD in the group without Gilbert syndrome (3.1 %). These data are highly clinically relevant since the prevalence of Gilbert syndrome is between 2-12 % in the general population, in the Czech Republic being 6.7 % (our unpublished data). Apart from being protected from atherosclerotic (and some other oxidative stress-mediated diseases), subjects with Gilbert syndrome are at higher risk of development of the pigment gallstone disease, and more importantly, clinically relevant drug intractions. These are linked to Gilbert syndrome genotype (mostly UGT1A1*28 allele homozygosity in Caucasian population) having twice as high prevalence compared to Gilbert syndrome phenotype due to low penetrance of this mutation.

A year later, we performed a meta-analysis of eleven studies covering male subjects, which demonstrated a strong negative relationship between serum bilirubin levels and severity of atherosclerosis in men ($p<0.0001$) (Novotny and Vitek 2003). Non-parametric, regression, and stratified analyses all reliably demonstrated a negative relationship between serum bilirubin concentrations and atherosclerotic diseases. A serum bilirubin level of 10.0 μmol/l was found to be the cut-point for discrimination of cardiovascular risk in analyzed men (for physiological serum bilirubin concentrations see Table 1). Interestingly, hyperbilirubinemia due to underlying liver function (reflected by elevated liver function enzymes) deterioration did not result in protection against cardiovascular diseases (Novotny and Vitek 2003) and was shown also later to eliminate protective effects of bilirubin on all-cause mortality (see also below) (Fulks *et al.* 2009). Thus this fact must be taken into consideration when assessing the individual risk of a patient – and also accounts for U-shape relationship between serum bilirubin and cardiovascular diseases observed in some studies (Breimer *et al.* 1995). Data from our meta-analysis were confirmed also in a recent meta-analytic study in both men and women (Kunutsor *et al.* 2015), although another one revealed only a non-significant protective trend for increased bilirubin concentrations (Stender *et al.* 2013).

Table 1. Physiological levels of serum bilirubin.

	Serum bilirubin (μmol/l)
Physiological range	3-17
Czech population	9.8* [8-12.4]
Gilbert syndrome	17-86

* Data represent median and IQ range and are based on our unpublished data of 717 subjects retrieved from WHO MONICA study.

We were also interested, whether serum bilirubin might be implicated in manifestation of premature coronary atherosclerosis. For that reason, we have studied 79 patients with premature myocardial infarction (defined

as its manifestation prior <45 and <55 years of age in males and females, respectively). Although these patients were found to also have lower bilirubin levels, to our surprise, the correlation of premature coronary artery disease with serum bilirubin was much milder that of patients with chronic IHD suggesting that other exogenous factors (e.g. smoking) as well as specific genetic predictors seem to be more important in manifestation of premature atherosclerosis (unpublished data).

We also put our focus on possible relationship between clinical markers of atherosclerotic lesions and systemic bilirubin concentrations. A cohort of 111 healthy men, 38 of whom had Gilbert syndrome, were analyzed for presence of carotid atherosclerosis, carotid intima-medial thickness of 0.8 mm being set as a marker of clinically relevant atherosclerosis (Vitek *et al.* 2006). Those subjects having mild elevation of serum bilirubin levels (benign hyperbilirubinemia) had very low prevalence of carotid atherosclerosis, and the negative relationship was significant even after adjustment for other risk factors (Vitek *et al.* 2006). Interestingly, the anti-atherogenic association of serum bilirubin was much more evident in older subjects (>48 years), and it was even possible to predict that the development of clinically relevant carotid atherosclerosis in subjects with Gilbert syndrome is postponed by almost 25 years. Our data are consistent also with majority of published reports on the association between serum bilirubin and peripheral artery disease. To cite at least one of the most important studies (in complex reviewed by Schwertner and Vitek 2004); in 2008, Perlstein *et al.* published a retrospective study on more than 7,000 adults from the National Health and Nutrition Examination Survey (NHANES) on this association. After adjustment for possible confounding factors, each 0.1 mg/dl (1.7 μmol/l) increase in serum bilirubin level was associated with 6 % reduction in the odds of having peripheral atherosclerosis.

Taking into account the above given data on atheroprotective effects of bilirubin, it is not surprising that identical negative correlations are present also for other diseases commonly associated with accelerated atherosclerosis. In fact, this is true for diabetes mellitus (for comprehensive review on the relationships between bilirubin and diabetes see Vitek (2012)). In our own, so far unpublished study on more than 200 patients with type 2 diabetes mellitus and almost 500 age-matched healthy controls, we were able to demonstrate much lower serum bilirubin levels in both male and female diabetic patients, and each micromolar increase of serum bilirubin was associated with substantial decrease of the odds of developing diabetes (Jiraskova 2011a). Our data are consistent with the majority of published data including a recent systematic review and meta-analysis demonstrating negative association between systemic bilirubin levels and type 2 diabetes mellitus, as well as metabolic syndrome (Nano *et al.* 2016). It is interesting to note that serum bilirubin levels were negatively associated with markers of glucose metabolism also in patients with type 1 diabetes mellitus (Mianowska *et al.* 2014). It is then logical, that the same negative relationship was found for serum bilirubin levels and obesity (Andersson *et al.* 2009), reviewed in Wagner *et al.* (2015), and beneficial role of bilirubin for serum lipid metabolism has also been consistently demonstrated (Bulmer *et al.* 2013). These associations are complex and involve negative association of serum bilirubin with total and LDL cholesterol and positive association with HDL cholesterol (Bulmer *et al.* 2013). In this respect, it is interesting to note that HDL is a potent binding molecule for bilirubin (apart from albumin) (Vitek and Ostrow 2009).

Bilirubin as an anti-atherogenic molecule – lessons from the clinical studies on diseases associated with increased risk of atherosclerosis

A wide array of various pathogenically different diseases is associated with increased risk of atherosclerosis. We have systematically studied some of them with remarkable and consistent findings of negative associations between the manifestation of these diseases and systemic bilirubin levels.

We studied a cohort of more than 50 patients with a Fabry disease, an X-linked metabolic disorder, caused by deficiency of the lysosomal enzyme α-galactosidase A resulting in accumulation of glycosphingolipids in endothelial cells and other tissues and organs (Zarate and Hopkin 2008). The majority of Fabry disease complications results from a vasculopathy associated with a progressive damage of the heart and vessels resulting in accelerated IHD (Anastasakis *et al.* 2013). In our study, Fabry disease was associated with significantly lower serum bilirubin levels and markers of increased oxidative stress. Interestingly, specific enzyme replacement therapy normalized both serum bilirubin levels as well as total serum antioxidant capacity suggesting that low serum bilirubin levels in this disease

are due to increased oxidative stress and enhanced consumption of endogenous antioxidants (Jiraskova *et al.* 2011b).

We were able to demonstrate similar associations for a variety of systemic autoimmune diseases. This was true for patients with Crohn´s disease (Lenicek *et al.* 2014), an inflammatory bowel disease often associated with increased cardiovascular risk (Wu *et al.* 2016). In this study, apart from much lower bilirubin concentrations in Crohn´s disease patients, Gilbert syndrome genotype (UGT1A1*28 homozygosity) was associated with a significant delay in Crohn´s disease manifestation (Lenicek *et al.* 2014).

Similarly, in our another study on patients with systemic lupus erythematosus (SLE), a systemic autoimmune disease associated with markedly increased risk of cardiovascular diseases (Lewandowski and Kaplan 2016), a significantly lower bilirubin levels were observed in these patients (Schwertner and Vitek 2004, Vitek *et al.* 2010a). Furthermore, each micromolar decrease in serum bilirubin was associated with a 37 % increase in the odds for a positive SLE status. Simultaneously, the odds of unconjugated hyperbilirubinemia characteristic for Gilbert syndrome were more than four times lower in SLE patients (Schwertner and Vitek 2004, Vitek *et al.* 2010a).

Similar and consistent results were found also on the relationship between serum bilirubin and the risk of rheumatoid arthritis in our clinical trial (Schwertner and Vitek 2004) and a study by Fishman *et al.* (2010), as well as between serum bilirubin and the risk of Wegener granulomatosis (our unpublished data), both autoimmune diseases being markedly associated with increased cardiovascular risk (Liao 2017, Faurschou *et al.* 2009, respectively).

Interestingly, similar negative association between serum bilirubin was observed also in patients with manifesting schizophrenia, a psychiatric disease linked to increased cardiovascular risk (Davidson 2002). In this study, the odds for unconjugated hyperbilirubinemia >17 μmol/l characteristic for Gilbert syndrome were four times lower in schizophrenic patients compared to the control population. Furthermore, each micromolar increase in serum bilirubin was associated with a 19 % decrease in the odds for schizophrenia status (Vitek *et al.* 2010b).

Finally, substantially lower serum bilirubin levels were detected in patients with colorectal cancer; each micromolar decrease in serum bilirubin being associated with a significant 7 % increase of colorectal cancer risk (Jiraskova *et al.* 2012). The link between colon cancer and atherosclerosis is not direct, but colon cancer is associated with metabolic syndrome and atherogenic lipid profile (Liu *et al.* 2010), both condition deeply involved in the process of atherogenesis.

To completely disclose the potential impact of systemic bilirubin levels on manifestation of various systemic diseases, studies by other authors should be mentioned. These include chronic obstructive pulmonary diseases (COPD) (Horsfall *et al.* 2011), psoriasis (Balta *et al.* 2014), multiple sclerosis (Peng *et al.* 2011), osteoporosis (Bian *et al.* 2013), and preeclampsia (Breslin *et al.* 2013). Surprisingly, all these conditions have been associated with an increased cardiovascular disease morbidity (Anagnostis *et al.* 2009, Coumbe *et al.* 2014, Ghoorah *et al.* 2013, Christiansen *et al.* 2010, McDonald *et al.* 2013) indicating a possible common role of bilirubin in protection against these diseases.

Relationship between serum bilirubin and markers of oxidative stress and inflammation

To explore what is the underlying link between bilirubin and decreased risk of cardiovascular diseases, we and others have clinically investigated the relationship of the markers of oxidative stress, inflammation and vascular dysfunction.

In one of our clinical studies on Gilbert syndrome subjects, we demonstrated in these hyperbilirubinemic individuals significantly lower serum levels of pentosidine and N^{ε}-carboxymethyl lysine, both belonging to advanced glycation end-products (AGEs), being positively associated with endothelial dysfunction, progression of atherosclerosis (Peppa *et al.* 2004) and diabetes (Turk *et al.* 2003). Levels of both AGEs remained substantially lower even after adjustment for selected vascular risk and other modifying factors (Kalousova *et al.* 2005). In another study on subjects with Gilbert syndrome we reported markedly lower urinary excretion of biopyrrins (Vitek *et al.* 2007a), tripyrrolic compounds produced by bilirubin oxidation under conditions of increased oxidative stress, including cardiovascular diseases (Hokamaki *et al.* 2004). These data were fully in line with our additional observation of negative association of serum bilirubin with systemic levels of 7-oxocholesterol and 7β-hydroxycholesterol (Vitek *et al.* 2013), both oxysterols being strongly correlated with vascular diseases (Vejux and Lizard

2009). In this study, serum bilirubin was also significantly correlated with total plasma peroxyl scavenging activity. It is noteworthy to mention, that we observed similar positive association of total serum antioxidant capacity (measured in ABTS® assay) also in our original cohort of Gilbert syndrome individuals (Vitek *et al.* 2002). An addition of bilirubin to the serum with a defined total antioxidant capacity led to a significant elevation of this parameter in response to a progressive increase of the bilirubin concentration. Interestingly, this increase was much higher than might be expected from stochiometric calculations supporting the existence of bilirubin/biliverdin redox cycle or another antioxidant defense amplifying mechanism (Vitek *et al.* 2002).

The same method for determination of total antioxidant capacity was applied also to much larger population of healthy subjects (n=288). Subjects in the lowest bilirubin quartile had apparently lower serum total antioxidant capacity as compared to subjects with highest serum bilirubin (P<0.0001). Importantly, subjects with the lowest serum bilirubin had also distinctly higher serum hsCRP levels as compared to subjects in the highest bilirubin quartile (Vitek *et al*. 2007b). The same relationship was reported also by other authors (Hwang *et al.* 2011). It should also be noted, that all these observations may account for improved endothelial function of subjects with mild elevation of unconjugated bilirubin, either in the form of Gilbert syndrome (Maruhashi *et al.* 2012), or iatrogenically induced (Dekker *et al.* 2011).

Other aspects of bilirubin protective action

There are many other possible aspects how bilirubin might exert its protective action, whose description is beyond the scope of this paper and which were subject of recent reviews. These include the beneficiary role of bilirubin on lipid metabolism (Bulmer *et al.* 2013), its anti-aging effects (Wallner *et al.* 2013a), potential anti-thrombotic effects *via* inhibition of platelet activation (Kundur *et al.* 2015), strong immunosupressive activities of bilirubin effects acting on almost all levels of both innate as well as adaptive immune response (Jangi *et al.* 2013), or even anti-mutagenic and anti-genotoxic effects of bilirubin (Molzer *et al.* 2013, Wallner *et al.* 2013b). As already mentioned above, bilirubin is even likely to activate PPARα nuclear receptors (Stec *et al.* 2016) further enlarging its potential to affect metabolic functions in a human body (Hinds *et al.* 2016) and possibly accounting for many of the clinical observations.

Bilirubin and overall mortality

Based on strong negative clinical associations described above, it is not surprising that systemic bilirubin concentrations are related cardiovascular, as well as all-cause mortality. Indeed, a large number of epidemiological studies investigating relationship of bilirubin and mortality have been reported during the last decade (for comprehensive review of this topic see Wagner *et al.* 2015). Among the largest studies was that by Fulks *et al.* (2009), who included nearly two million health insurance applicants and investigated all-cause mortality associated with bilirubin concentrations. In this study, lower bilirubin levels were associated with significantly increased mortality. Significant negative association of serum bilirubin concentrations and overall mortality was observed also in a recent US NHANES study on older adults above 60 years of age (Ong *et al.* 2014). And finally, in their large studies Horsfall *et al.* (2012) confirmed enhanced mortality rates in subjects with lower bilirubin concentrations, whereas subjects with Gilbert syndrome had twice as low mortality compared to normobilirubinemic subjects (Horsfall *et al.* 2013).

Could bilirubin be a therapeutic target?

This is a question intensively studied over the last years, and comprehensively reviewed in recent literature (McCarty 2007). Based on data described in this paper it is important to emphasize, that even tiny (i.e. single units of micromoles/l) elevations of systemic (and probably also only topical) concentrations of bilirubin can produce substantial benefit resulting in significantly decreased risk of many oxidative stress-mediated diseases. Such elevation might be achieved by induction of heme oxygenase (Muchova *et al.* 2007), the key enzyme degrading heme to biliverdin/bilirubin, or inhibition of bilirubin UDP-glucuronosyl transferase (UGT1A1), the key enzyme responsible for bilirubin conjugation in the liver tissue (Dekker *et al.* 2011). It is also likely that many drugs commonly used in clinical medicine may exert these activities and result in mild elevation of serum bilirubin levels, statins being such an example (Vitek *et al.* 2011). Moreover, other tetrapyrrolic molecules related to

bilirubin/biliverdin molecular structure occurring commonly in nature may exert similar effect as bilirubin. Such an example might be phycocyanobilin, a linear tetrapyrrolic molecule occurring in edible algae, which, at least experimentally, was demonstrated to delay progression of atherosclerosis activating several atheroprotective mechanisms (Strasky *et al.* 2013).

Conclusion

Increasing pile of evidence suggests that bilirubin is a highly bioactive molecule having deep impact on prognosis of cardiovascular and other diseases. Due to recent discoveries of bilirubin binding to nuclear receptors (besides PPARα, aryl hydrocarbon receptor (AhR) is another typical nuclear bilirubin receptor), and taking into account its production in remote organs, bilirubin behaves in certain circumstances as a hormone. It is interesting to note that similar endocrine effects have been recently discovered for bile acids, also considered for long time negligible molecules in this regard (Vitek and Haluzik 2016). Apart from known antioxidant and other biological activities of bilirubin it seems likely that its "endocrine" effects importantly contribute to health-promotion and may account for many clinical observations. Although we still do not know all the molecular mechanisms behind its biological action, bilirubin becomes an important prognostic marker and potential therapeutic target.

Conflict of Interest

There is no conflict of interest.

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Abbreviations

AGEs, advanced glycation-end products; HDL, high density lipoprotein; hsCRP, highly sensitive C-reactive protein; IHD, ischemic heart disease; LDL, low density lipoprotein; SLE, systemic lupus erythematosus; UGT1A1, bilirubin UDP-glucuronosyl transferase.

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