Bilirubin, The Gold Within

Running title: Seppen et al.; Bilirubin, the gold within

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When heme containing proteins such as hemoglobin and cytochrome P450 are degraded, their prosthetic groups cannot be metabolised and have to be excreted. Elimination of heme could be considered straightforward detoxification but a large body of evidence shows that this pathway plays a role in various physiological processes as well.

The first step in the catabolism of heme is cleavage of the porphyrin ring by heme oxygenase, this reaction yields biliverdin, carbon monoxide and Iron. Two heme oxygenase (HO) isoforms mediate this process, constitutively expressed HO-2 and inducible HO-1. Many cell types express heme oxygenase, with high expression present in cells of the reticuloendothelial system involved in degradation of erythrocytes\(^1\). The first step in heme catabolism has attracted considerable interest since heme oxygenase activity is involved in the regulation of processes such as artherosclerosis, inflammation and diabetes\(^1\). This regulation is mediated by the products of heme oxygenase action on heme, with the potent signalling molecule carbon monoxide likely playing a major role\(^1\).

The other product of heme oxygenase action on heme is the green and water soluble compound biliverdin. In mammals, levels of biliverdin are low because it is quickly converted to the hydrophobic yellow compound bilirubin by ubiquitously expressed biliverdin reductase. However, other animal species such as fish\(^2\), do not complete metabolize all biliverdin to bilirubin and excrete significant amounts of the green pigment. Because bilirubin is poorly water soluble, mammals require efficient conjugation with glucuronic acid by UDP glucuronyltransferase before bilirubin glucuronides can be excreted into bile. This aspect of heme metabolism has always puzzled scientists. Why is bilirubin the sole end product of heme degradation in mammals? Bilirubin is toxic, the absence of bilirubin UDP glucuronyltransferase (UGT1A1) in patients with Crigler-Najjar syndrome causes high unconjugated bilirubin levels
and subsequent severe brain damage. In addition, the conjugation of bilirubin is an energy requiring process whereas biliverdin is water soluble and can be directly eliminated into bile.

One of the most compelling theories is that evolution of the placenta created the need for complete conversion of biliverdin to bilirubin in mammals. Several studies have shown that bilirubin is able to traverse the placenta, either by diffusion or transport, whereas biliverdin cannot\(^3\). Thus, the complete conversion of biliverdin to bilirubin would be a mechanism by which the developing fetus can get rid of heme metabolites\(^3\).

Arguing against this hypothesis is the recent discovery that humans homozygous for mutations that inactivate biliverdin reductase do not appear to present with physical problems\(^4\). The absence of biliverdin reductase should prevent the conversion of biliverdin to bilirubin and indeed, during periods of obstruction of bile flow, plasma of these subjects became bright green. However, although the absence of biliverdin reductase gave rise to elevated biliverdin levels, plasma of these people also contained bilirubin\(^4\) suggesting an alternative route of biliverdin conversion.

Molecular biology offers another line of evidence for a beneficial role of bilirubin. Around 10% of the population has Gilbert syndrome, characterised by mildly elevated plasma bilirubin\(^5\). In Caucasians, Gilbert is caused by a TA polymorphism in the promoter region of UGT1A1, the gene encoding bilirubin UDP glucuronyltransferase.\(^6\) Homozygotes for this UGT1A1*28 polymorphism have serum bilirubin levels in the high normal range but combination with other factors, such as increased hemolysis, will elevate bilirubin levels further. The high frequency of the UGT1A1*28 polymorphism causing elevated bilirubin suggests a positive evolutionary selection for this allele. Compelling evidence that slightly elevated bilirubin levels confer an evolutionary advantage comes from studies in different ethnic groups.
In Asians, mildly elevated bilirubin levels are also prevalent but the molecular mechanism behind this elevation is different. Whereas in Caucasians Gilbert is dependent on the UGT1A1*28 polymorphism, in Asians a G>A mutation in nucleotide 211 of the coding region of the UGT1A1 gene is the main cause of Gilberts\(^7\). Two distinct molecular mechanisms causing the same phenotype suggest convergent evolution of the same trait and thus provide strong evidence for an evolutionary advantage of elevated bilirubin levels. However, it is also possible that elevated bilirubin levels are not beneficial but that reduced glucuronidation by UGT1A1 of an as yet unidentified compound provides a selective advantage. In this scenario, elevated bilirubin levels would just be an epi phenomenon. Glucuronidation by UGT1A1 is also important for the detoxification of hormones such as estradiol\(^8\). Because it is easily imaginable that elimination speed of hormones affects fertility, the possibility that the evolutionary advantage of Gilberts polymorphisms have nothing to do with bilirubin cannot be completely discarded.

On the other hand, several studies have reported an association between low serum bilirubin levels and increased risk for Cardio Vascular Disease (CVD) thus suggesting a health benefit of elevated bilirubin levels. A large study published in this issue of Circulation adds to this body of evidence and for the first time also shows the association between low bilirubin and increased risk in both males and females\(^9\). Because CVD typically presents at an age where reproduction has already taken place, a protective effect of bilirubin against CVD is a less likely explanation for the evolutionary benefits of bilirubin and high frequency of Gilberts alleles. Therefore, the selective pressure by which mammals evolved to completely convert biliverdin to bilirubin and the maintainance of the high frequency of Gilberts alleles in the human population must still be identified.
Notwithstanding these evolutionary considerations, with the publication of the study by Horsfall in this issue of Circulation, the evidence that low bilirubin is associated with elevated risk for CVD is now convincing. The mechanism behind this association is still not entirely clear and a causal association between serum bilirubin and heart disease has yet to be established. A direct effect of bilirubin seems likely but other parts of the heme catabolism might also play a role.

The most straightforward explanation for the observed protection of bilirubin against CVD is the antioxidant capacity of bilirubin that has been known for many years \(^\text{10}\). Bilirubin effectively suppresses the oxidation of lipids and lipoproteins and has been suggested to be a natural antioxidant.

In view of the compelling data underscoring the role of oxidative stress in the pathogenesis of CVD, a causal relationship between lower serum levels of the anti-oxidant bilirubin and an increased risk for CVD is likely. Basic studies indeed indicate that enhancing anti-oxidant levels, for instance by increasing serum bilirubin levels, can be beneficial. Several large trials however, using oral administration of anti-oxidants like b-carotene, vitamin C and E, had no effect or even resulted in an increased mortality by CVD \(^\text{11}\). Thus, clinical evidence that anti-oxidants reduce the risk of CVD events is still lacking.

These observations raise the question if the anti-oxidant properties of bilirubin are responsible for the apparent protective effect of bilirubin on CVD. To find an explanation for the association of bilirubin levels and CVD we should take a closer look at the three steps involved in bilirubin metabolism.

Heme oxygenase 1 (HO-1) catalyzes the first step of heme catabolism which converts heme into biliverdin while releasing carbon monoxide and iron. Induction of HO-1 in animals is protective against arteriosclerosis \(^\text{12,13}\). In man, the magnitude of this protective induction
depends on the length of a GT nucleotide repeat in the HO-1 promoter, shorter repeats result in a stronger induction. Several studies have demonstrated the association of this polymorphism with CVD risk, thus suggesting a protective effect of the shorter GT repeats that result in higher HO induction\textsuperscript{14}. Presence of the shorter repeat has a marginal effect on serum bilirubin levels and induction of HO-1 with curcumin or resveratrol does not increase bilirubin levels either. Niacin mediated induction of HO-1 does increase serum bilirubin but this could also be explained by increased phagocytosis of erythrocytes. Although these data indicate a minor role for HO-1 activity in determining serum bilirubin concentration, induction of HO-1 will increase local levels that can protect against inflammation and oxidation.

It should be kept in mind that HO activity also releases the potent signalling molecule CO, since ample evidence shows the involvement of CO in cardiovascular function, it is not yet clear whether the effects of induced HO are due bilirubin, CO or both.

Biliverdin reductase converts biliverdin to bilirubin. It has been suggested that this provides a cellular redox cycle where bilirubin acts as a scavenger for radicals with biliverdin as the end product\textsuperscript{15}. Intracellular biliverdin reductase would subsequently regenerate bilirubin and maintain antioxidant status. However, since oxidation of bilirubin only yields low amounts of biliverdin and overexpression of biliverdin reductase does not increase resistance towards oxidative stress, a direct role of this enzyme in the protection against CVD does not seem likely\textsuperscript{16}.

Glucuronidation by UGT1A1 has a controlling effect on serum bilirubin levels\textsuperscript{5}. Since the UGT1A1*28 Gilberts polymorphism results in higher (protective) serum bilirubin levels, it is expected to be associated with reduced CVD risk. Seven studies have addressed this and all confirmed the association of UGT1A1*28 with higher bilirubin levels\textsuperscript{17}. Five retrospective
studies however did not reveal an association of this polymorphism with a decreased risk for CVD, while two did. The strongest protective effect of elevated bilirubin on CVD was reported in a prospective population based cohort study. This last study differed from all other studies because subjects with a lower mean age were included (36 versus 60 > years). The loss of subjects with a high CVD risk from the older study groups may result in a survival bias. Furthermore, serum bilirubin levels increase with age while only levels <10uM show an increased CVD risk. Therefore, the gradual increase in serum bilirubin may compromise the association in older populations. However, even in the young cohort, the association of serum bilirubin and CVD was stronger than that of the UGT1A1 polymorphism. Thus, although in this population reduced UGT1A1 activity seems protective, serum bilirubin levels better predict CVD risk.

Together, this evidence indicates that other factors apart from UGT1A1 genotype play a role in the association between reduced bilirubin serum levels and an increased risk of CVD. It is unquestionable that bilirubin is a potent antioxidant, bilirubin consumption by oxidative processes will therefore reduce its serum level. The association of low serum bilirubin with CVD risk could therefore also be due to increased bilirubin oxidation in subjects at risk. Thus, this association could be a reflection of underlying pathological oxidative processes at the root of CVD.

The reduced bilirubin levels in smokers clearly demonstrate the potential impact of oxidative stress. This is underscored by the association between serum and urine levels of oxidative metabolites of bilirubin with mortality in acute myocardial infarction. Therefore, measuring bilirubin oxidation products seems needed to clarify the potential role of oxidative stress in the association between serum bilirubin and CVD.
In conclusion, the large study by Horsfall and collaborators now demonstrates that the association between reduced serum bilirubin levels and increased CVD risk is strong in both sexes. It seems likely that several factors are playing a role, antioxidant effects of bilirubin, heme oxygenase activity and consumption of bilirubin by oxidative processes could all be involved. Resolving the exact mechanism behind this association is therefore needed to establish the relevance of these factors and to allow a rational design of a treatment aimed at reducing the CVD risk in subjects with a low serum bilirubin.

**Conflict of Interest Disclosures:** None.

**References:**


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