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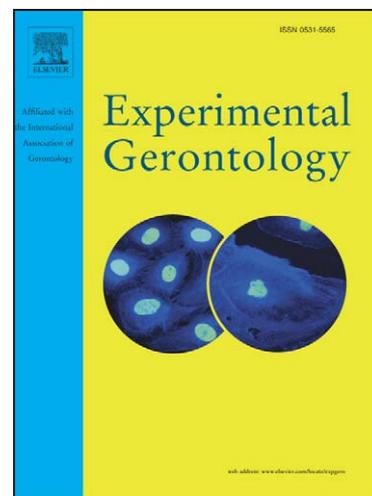
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Serum bilirubin levels, *UGT1A1* polymorphisms and risk for coronary artery disease

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Abstract

Low levels of the antioxidative serum bilirubin are associated with vascular aging and an increased risk for coronary artery disease (CAD). *UGT1A1* is the major gene influencing bilirubin concentrations. Therefore, we investigated an association of bilirubin levels and two polymorphisms in the promoter of *UGT1A1* [-53(TA-repeat) polymorphism and T-3279G] in 477 patients with premature, familial CAD and 619 age- and sex-matched controls. Bilirubin concentrations were significantly lower in cases than in controls (0.62 ± 0.36 vs. 0.76 ± 0.41 mg/dl for men, $p=1.2\times 10^{-10}$; and 0.42 ± 0.29 vs. 0.55 ± 0.23 mg/dl, $p=1.9\times 10^{-9}$ for women). Both polymorphisms showed a strong association with bilirubin levels with higher levels for homozygote carriers of the minor allele. These associations were most pronounced in male controls and patients ($p=5.9\times 10^{-26}$ and $p=3.4\times 10^{-16}$, respectively, for the -53(TA-repeat) polymorphism). Logistic regression analysis revealed low bilirubin levels but not the *UGT1A1* polymorphisms to be significantly associated with CAD: OR (95%CI) 0.90 (0.86-0.94), $p=2.6\times 10^{-6}$ for men and 0.77 (0.68-0.87), $p=3.2\times 10^{-5}$ for women, respectively for each 0.1 mg/dl increase of bilirubin. These results indicate that it is rather decreased bilirubin levels in general than the changes in the genetic variation of this gene that increase the risk for CAD.

Keywords: bilirubin, coronary artery disease, *UGT1A1*, association study, polymorphism

1. Introduction

Several studies have shown that bilirubin, a major product of heme catabolism, has a potent antioxidative capacity (Stocker et al., 1987; Neuzil et al., 1994). Animal experiments in congenitally hyperbilirubinemic Gunn rats with a defective uridine-diphosphate-glucuronosyltransferase compared to wildtype rats demonstrated virtually no neointimal proliferation after intimal injury caused by balloon angioplasty. When wildtype rats were pretreated with the bilirubin precursor biliverdin before injury, neointimal formation was also significantly reduced. Cultured vascular smooth muscle cells from rats showed a significantly reduced proliferation when treated with bilirubin (Öllinger et al., 2005). These properties and bilirubin's ability to scavenge peroxy radicals indicates a protective role of bilirubin against vascular aging and associated atherosclerotic diseases (Schwertner et al., 2008). This is in line with epidemiological studies reporting low serum bilirubin levels to be associated with an increased risk for coronary artery disease (CAD) or other atherosclerosis outcomes (Schwertner et al., 1994; Hopkins et al., 1996; Hunt et al., 2001; Novotny et al., 2003; Djousse et al., 2003; Endler et al., 2003; Schwertner et al., 2000; Breimer et al., 1995; Levinson 1997; Troughton et al., 2007; Hunt et al., 1996; Lin et al., 2006; Rantner et al., 2008). A study in healthy individuals grouped by low, intermediate and high serum bilirubin levels revealed that elevated bilirubin concentrations protect from coronary flow reserve impairment, coronary microvascular dysfunction, and possibly coronary atherosclerosis (Gullu et al., 2005).

Two recent genome-wide linkage studies suggested *UGT1A1*, the gene encoding for uridine-diphosphate-glucuronosyltransferase, as a major locus for serum bilirubin levels (Kronenberg et al., 2002; Lin et al., 2003). The gene product of *UGT1A1* catalyzes the conjugation of serum bilirubin to facilitate its removal by biliary excretion. Polymorphisms in this gene result in a decreased capacity to glucuronidate bilirubin, as observed in Gilbert syndrome and some forms of perinatal jaundice (Clarke et al., 1997; Maruo et al., 1999). A common polymorphism in the promoter of *UGT1A1* with five to nine TA-repeats in the TATA box at position -53 was reported to show an inverse association with the transcriptional activity (Bosma et al., 1995). So far five different alleles with (TA)₅₋₉-repeats have been described with (TA)₆ and (TA)₇ repeats as the main alleles with a frequency of up to 60% and 40%, respectively, in

Caucasian populations (Beutler et al., 1998). Functional studies demonstrated that carriers of an allele with (TA)₇-repeats show a lower transcriptional efficiency and a decrease of uridine-diphosphate-glucuronosyltransferase activity of about 70% compared to those with less than 7 repeats (Bosma et al., 1995). This reduced enzyme activity causes increased unconjugated bilirubin concentrations in heterozygous and homozygous carriers of the (TA)₇-repeats.

A recent study in 11 Japanese and 12 Caucasian patients with Gilbert's syndrome reported homozygosity for both, the *UGT1A1* -53(TA)₇ repeat allele and the G allele of the T-3279G (rs4124874) polymorphism. The T-3279G polymorphism is located in a phenobarbital-responsive enhancer element in the 5' region of the gene. The presence of the -3279G allele results in a lower transcriptional activity, a reduced conjugation of bilirubin and higher serum bilirubin concentrations (Maruo et al., 2004).

Due to the contradictory results of association studies investigating the *UGT1A1* TA-repeat polymorphism and atherosclerotic complications (Bosma et al., 2003; Lin et al., 2006; Gajdos et al., 2006; Rantner et al., 2008) we decided to investigate the association of this and another recently described promoter polymorphism with bilirubin levels as well as CAD in a large group of patients with premature familial CAD in comparison to a control group.

2. Patients and Methods

2.1 Patients and Controls

This case-control study is based on 1096 individuals of European ancestry from the same geographical region of Utah who are known to have little racial admixture. The 477 patients (365 men and 112 women) with premature, familial CAD had survived a myocardial infarction, percutaneous transluminal angioplasty, or coronary artery bypass grafting before age 55 for men or age 65 for women. To minimize artifactual effects of the acute coronary syndrome on biochemical measurements, patients were seen at least 6 months after their acute event. Each of the CAD cases was from a family in which at least one additional first-degree relative had early CAD by this definition. The control group is based on 833 individuals representing a general population sample from the same geographical region as described recently (Schoenborn et al., 2006). From this control group we selected 397 men and 222

women who represented a similar age distribution as the respective gender-specific patient groups and which can be considered as an approximate 1:1 matching for men and 1:2 matching for women (patients:controls). Ethical approval by local committee and written informed consent was obtained from each participant.

2.2 Bilirubin measurement

All biochemical measurements were made in overnight fasting venous samples. Total serum bilirubin was measured by a thin film adaptation of a diazonium salt, colorimetric method using the Vitros analyzer (Ortho-Clinical Diagnostics, Inc. Rochester NY 14650).

2.3 Genotyping

The UGT1A1 -53(TA-repeat) promoter polymorphism was analyzed by the ABI 3130xl sequencing system exactly as described recently (Lin et al., 2006). The -3279T>G polymorphism (rs4124874) was determined by use of a 5'-Nuclease Assay and the ABI Prism® 7900HT system. Additionally 10 % of the samples were genotyped in duplicate for quality control. Genotyping was performed within the Genotyping Unit of the Gene Discovery Core Facility at the Innsbruck Medical University, Austria.

2.4 Statistical analysis

To compare characteristics of patients and controls, we applied unpaired t-tests nonparametric Wilcoxon tests and Pearson's χ^2 -test. The genotype frequencies for cases and controls of both genders were in Hardy-Weinberg equilibrium for both polymorphisms. Linkage disequilibrium between the two polymorphisms was assessed by the correlation coefficient r and Lewontin's D' . Haplotypes based on the two SNPs were estimated via the expectation-maximization algorithm by the SAS® procedure PROC HAPLOTYPE (version 9.1, release 2004). Differences between bilirubin levels between the genotype and haplotype groups stratified for case-control status or between controls and CAD cases stratified for genotype groups were tested using linear regression adjusting for age. An additive (i.e. estimating the change of mean bilirubin levels per minor allele) and a recessive (i.e. change of mean bilirubin levels of subjects with two copies of the minor allele versus the rest) genetic model was applied. Percentage of bilirubin variance explained by classical factors and by the two polymorphisms was estimated by the r^2 (Field 2005). Bilirubin concentrations

were analyzed on a transformed scale ($\ln(\text{bilirubin}+1)$) in order to obtain a normally distributed variable. Logistic regression was applied to estimate the association of bilirubin levels and the polymorphisms with the change of odds for CAD. Statistical analysis was performed with SPSS for Windows 15 and SAS (version 9.1, release 2004).

3. Results

3.1 Baseline characteristics

The demographic and laboratory parameters of the 477 CAD patients and the 619 controls are presented in Table 1. All analyses were stratified by gender. In both genders cases showed higher concentrations of total cholesterol, LDL-cholesterol, triglycerides, glucose and serum creatinine and lower levels for HDL-cholesterol and albumin concentrations as expected. A higher frequency of current smokers and ex-smokers was found among the cases. Serum bilirubin levels were about 20% lower in CAD patients compared to controls of both genders. Independent of case status, females exhibited lower bilirubin concentrations compared to males ($p<0.001$).

3.2 Genotype characteristics

Lewontin's D' of the -53(TA) and T-3279G locus was almost unity ($D'=0.9969$) with a correlation coefficient of $r=0.77$. There were no statistical significant differences in the genotype distribution when comparing men and women or when comparing cases and controls. The minor allele frequencies for the -53(TA-repeat) and the T-3279G polymorphism ranged between 30.7% and 31.9% and between 42.5 and 49.1%, respectively, in the four subgroups, male and female controls or patients (Table 2).

3.3 Bilirubin concentrations depend on UGT1A1 polymorphisms (analysis stratified by case-control status)

We observed strong associations between both polymorphisms and bilirubin levels in the case control-stratified analysis (Table 2). Homozygote carriers of the minor allele of the -53(TA-repeat) polymorphism showed the highest bilirubin concentrations. Heterozygote individuals showed bilirubin levels closer to the levels of homozygote wildtypes. These associations were very strong in the recessive model in male controls ($p=5.9 \times 10^{-26}$), male CAD patients ($p=3.4 \times 10^{-16}$) and female

CAD patients ($p=0.00001$), but were of borderline significance in female controls ($p=0.09$). Similar observations but with less strong effect sizes were observed for the T-3279G polymorphism (Table 2).

The total variation of bilirubin levels explained by the two polymorphisms combined was 15.4% in cases, and 13.6% in controls (Table 3). The analysis of the variation of bilirubin levels explained by the two single polymorphisms separately revealed that the T-3279G polymorphism explained less of the bilirubin variation than the -53(TA-repeat) polymorphism.

3.4 Bilirubin concentrations depend on CAD case-control status (analysis stratified by genotype groups)

When analyzing the genotype-specific differences in bilirubin levels between patients and controls, highly significantly lower bilirubin levels were observed among CAD patients compared to controls when focusing on subjects homozygous for the major allele (6/6 or T/T) or heterozygous (6/7 or T/G) (see most right column of Table 2 with p values ranging from 0.0004 to 1.0×10^{-7}). For subjects homozygous of the minor allele (7/7 or G/G), we observed a trend towards lower bilirubin levels in men ($p=0.06$ and 0.07 for the -53(TA-repeat) and the T-3279G polymorphism, respectively), but no difference in women ($p=0.15$ and 0.32, respectively).

3.5 Haplotype analysis

In concordance with the single polymorphism analysis, individuals carrying two copies of the major allele of both polymorphisms had the lowest bilirubin levels and haplotypes based on the minor allele at both SNPs were associated with the highest bilirubin levels (data not shown).

3.6 Association of bilirubin concentrations with CAD outcome, but no association of the polymorphisms with CAD outcome

After adjustment for age (model 1) and other risk factors (models 2 and 3), we observed a significant decrease in CAD risk per 0.1 mg/dl increase in serum bilirubin concentrations of about 6-10% in men and for about 25% in women (Table 4). No significant change in CAD risk depending directly on the genotype groups of the two polymorphisms was observed.

4. Discussion

4.1 Confirmed protective properties of bilirubin for CAD

The role of bilirubin in the pathogenesis of cardiovascular disease is still not entirely understood. The fact that it is antioxidative and cytoprotective suggests anti-atherogenic properties. As demonstrated, bilirubin has a comparable antioxidative capacity as vitamin E (Stocker et al., 1987) and alleviates the atherogenic stress of lipid peroxidation by preventing oxidative modification of LDL (Neuzil et al., 1994). Thus, serum bilirubin concentration itself and/or factors that modify its equilibrium are responsible for the protective properties. The present study in 477 CAD patients and 619 healthy controls confirmed the results of several previous epidemiological studies (Schwertner et al., 1994; Hopkins et al., 1996; Hunt et al., 2001; Novotny et al., 2003; Djousse et al., 2003; Endler et al., 2003; Schwertner et al., 2000; Breimer et al., 1995; Levinson 1997; Troughton et al., 2007; Hunt et al., 1996; Lin et al., 2006) that low serum bilirubin levels were associated with increased risk for coronary events.

4.2 Genetic variation within the *UGT1A1* gene and CAD

Two polymorphisms in the promoter region of the *UGT1A1* gene, the -53(TA-repeat) and the T-3279G polymorphism, were shown to be strongly associated with serum bilirubin levels (Bosma et al., 1995; Maruo et al., 2004). The presence of the -53(TA)₇ allele and the -3279G allele are known to have a reduced enzyme activity of the gene product and cause increased serum bilirubin concentrations (Bosma et al., 1995; Maruo et al., 2004). Our data clearly indicated a significant association of both polymorphisms with bilirubin levels, but no association of either of the polymorphisms with CAD status.

Meantime four other studies (Bosma et al., 2003; Gajdos et al., 2006; Lin et al., 2006; Rantner et al., 2008) investigated the association between the -53(TA-repeat) polymorphism and CHD. First, the prospective population-based Rotterdam Study, a nested case-control study including 185 CHD cases and 255 controls, found no protective effect of the -53(TA)_{7/7} genotype on CHD, although carriers of the -53(TA)_{7/7} genotype showed significantly increased bilirubin concentrations. However, this study also did not observe an association between low serum bilirubin and increased risk of CHD, in clear contrast to the majority of the literature. Second, from

the ECTIM Study including 366 male cases with myocardial infarction and 314 male controls, Gajdos and coworkers (Gajdos et al., 2006) obtained further evidence that the *UGT1A1* TA-7 allele is not associated with a decreased risk for myocardial infarction. Bilirubin concentrations were not available in that study. Third, a recent study in patients with peripheral arterial disease (PAD) revealed similar results as the study at hand, i.e. a clear association between low bilirubin concentrations and PAD but no association between the *UGT1A1* polymorphism and PAD. These three studies and the present study are in contrast to the results of the prospective Framingham Offspring Study, a 24-year follow-up study with 1780 participants (Lin et al., 2006) which found for the first time a significantly decreased risk of CVD and CHD for subjects with the -53(TA)_{7/7} genotype. Individuals with this genotype had about a third of the risk for CVD and CHD than those with genotype 6/6 or 6/7.

There are several explanations which might have contributed that we did not find an association of the *UGT1A1* polymorphisms with CAD and some of them were discussed in detail recently by Schwertner and Vitek (Schwertner et al., 2008). The first one is the case-control design of our study. Due to the fact that all included cases in our study had survived a CAD event for at least six months, we might speculate on a survival bias considering that about half of the incident cases do not survive the first half year of a CAD event and that the further survival after the first event is influenced by genetic factors (Marin et al., 2005). Given this hypothesis, the fact that we still detected the association of bilirubin levels with CAD might be explained by the strengths of this association and the situation that the *UGT1A1* polymorphisms explain only about 10-15% of the bilirubin variance (Table 3). An additional and more likely explanation could be the existence of other factors that increase CAD risk and decrease bilirubin levels and are responsible for the ~85-90% of the variance in bilirubin not explained by the two *UGT1A1* polymorphisms. The markedly lower bilirubin levels in CAD patients compared with controls within the genotype groups suggest such factors. These factors could have been confounding the gene-CAD association in our study. Assuming that bilirubin production is most likely affected by heme oxygenase, a further reason for the association of low bilirubin levels and an increased risk for CAD and might be the role of heme oxygenase-1 (HO-1) polymorphisms in the development of CAD (Morita 2005; Exner et al., 2004; Vitek et al., 2007). Another possible explanation is reverse causation,

meaning that the CAD status influences bilirubin levels rather than bilirubin levels modifying CAD risk. However, prospective studies rather indicate a truly causative effect of bilirubin on CAD with decreased levels long before the CAD event (Djousse et al., 2001; Breimer et al., 1995; Lin et al., 2006; Troughton et al., 2007). Given that the causative effect of bilirubin levels on CAD risk is true, the most likely explanation for not finding an *UGT1A1* association with CAD is a lack of power. If increased bilirubin levels cause a decreased risk of CAD of 10-25% per unit bilirubin, we have sufficient power to find this association. However, since the polymorphism shows various expressivity within each genotype group, the polymorphism explains only 10-15% of the variation in bilirubin levels. Therefore, our ability to detect this polymorphism-CAD association drops substantially if we consider also the fact that the frequency of the -53(TA)_{7/7} genotype in CAD patients was expected to be half of the controls in case of a power of 80%.

Under the hypothesis that the *UGT1A1* polymorphisms' changes decreased CAD directly, we would expect an equally strong or even stronger association of the polymorphism with CAD than the association of bilirubin levels with CAD. As this was not the case, we can conclude that it is low bilirubin levels in general that seem to increase CAD risk than just the *UGT1A1* polymorphisms. Interestingly, the recent genome-wide association study by the Wellcome Trust Case Control Consortium did not find a significant association of the *UGT1A1* gene region with CAD (The Wellcome Trust Case Control Consortium 2007).

4.3 Gender differences

Hunt and colleagues (Hunt et al., 2001) reported a borderline significant difference in serum bilirubin levels between CHD cases and controls in males ($p=0.08$) but no differences in females. Similar results were found by Endler et al. (Endler et al., 2003). Our study found an association of low bilirubin levels with CAD in both genders, which was even stronger in females: logistic regression analysis revealed a decreasing risk for CAD of about 10% and 25% for men and women, respectively, with each increase of bilirubin of 0.1 mg/dl. Similarly, Erdogan et al. (Erdogan et al., 2006) found an association in both, men and women. Reasons for these contrasting results might be the small number of women with the investigated outcomes and therefore low power or major age differences between women cases and controls. Interestingly, the association of the two investigated polymorphisms

with bilirubin levels was much smaller in female controls than in male controls but still very strong in both genders of cases (Table 2).

4.4 Limitations of the Study

We did not adjust our findings for liver enzymes since these parameters were not available in the entire group. However, when we performed a sensitivity analysis in those patients and controls with alkaline phosphatase (ALP), aspartate transaminase (AST), or gammaglutamyltransferase (GGT) available, we observed similar results as described above (data not shown).

A stratified analysis of subjects with hyperbilirubinemia above 1 mg/dL was not suitable since the frequency of this state was only 4.3% and 12.5% in females and males, respectively.

We did not investigate further polymorphisms in the region of UGT1A1. Hong et al. reported besides the TA repeat polymorphism 14 other SNPs and demonstrated that conditional on the TA repeat polymorphism no other variant was shown to be significantly associated with bilirubin in both genotype and haplotype analyses (Hong et al., 2007). According to this thorough study, we have already tagged the common haplotypes with the two polymorphisms investigated in our study. If there is indeed an additional variant which contributes to bilirubin levels independent from the TA repeat polymorphism, it can be expected that the effect is quite small which would require a large sample size.

4.5 Conclusion

In summary, the present case-control study showed a strong and consistent association of low serum bilirubin levels with CAD for both genders. Despite a clear association of the two analyzed *UGT1A1* promoter polymorphisms with bilirubin concentrations, we could not detect a significant association of these polymorphisms with CAD indicating that it is rather decreased bilirubin levels in general than the changes in the gene product caused by the particular polymorphisms that increase the risk of CAD.

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ACCEPTED MANUSCRIPT

References

1. Beutler, E., Gelbart, T., Demina, A., 1998. Racial variability in the UDP-glucuronosyltransferase 1 (UGT1A1) promoter: a balanced polymorphism for regulation of bilirubin metabolism? *Proceedings of the National Academy of Sciences of the United States of America* 95, 8170-8174.
2. Bosma, P.J., Chowdhury, J.R., Bakker, C., Gantla, S., de Boer, A., Oostra, B.A. et al., 1995. The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *New England Journal of Medicine* 333, 1171-1175.
3. Bosma, P.J., van, d.M., I, Bakker, C.T., Hofman, A., Paul-Abrahamse, M., Witteman, J.C., 2003. UGT1A1*28 allele and coronary heart disease: the Rotterdam Study. *Clinical Chemistry* 49, 1180-1181.
4. Breimer, L.H., Wannamethee, G., Ebrahim, S., Shaper, A.G., 1995. Serum bilirubin and risk of ischemic heart disease in middle-aged British men. *Clinical Chemistry* 41, 1504-1508.
5. Clarke, D.J., Moghrabi, N., Monaghan, G., Cassidy, A., Boxer, M., Hume, R. et al., 1997. Genetic defects of the UDP-glucuronosyltransferase-1 (UGT1) gene that cause familial non-haemolytic unconjugated hyperbilirubinaemias. *Clinica Chimica Acta* 266, 63-74.
6. Djousse, L., Levy, D., Cupples, L.A., Evans, J.C., D'Agostino, R.B., Ellison, R.C., 2001. Total serum bilirubin and risk of cardiovascular disease in the Framingham offspring study. *American Journal of Cardiology* 87, 1196-1200.
7. Djousse, L., Rothman, K.J., Cupples, L.A., Levy, D., Ellison, R.C., 2003. Effect of serum albumin and bilirubin on the risk of myocardial infarction (the Framingham Offspring Study). *American Journal of Cardiology* 91, 485-488.
8. Endler, G., Hamwi, A., Sunder-Plassmann, R., Exner, M., Vukovich, T., Mannhalter, C. et al., 2003. Is low serum bilirubin an independent risk factor for coronary artery disease in men but not in women? *Clinical Chemistry* 49, 1201-1204.
9. Erdogan, D., Gullu, H., Yildirim, E., Tok, D., Kirbas, I., Ciftci, O. et al., 2006. Low serum bilirubin levels are independently and inversely related to impaired flow-mediated vasodilation and increased carotid intima-media thickness in both men and women. *Atherosclerosis* 184, 431-437.
10. Exner, M., Minar, E., Wagner, O., Schillinger, M., 2004. The role of heme oxygenase-1 promoter polymorphisms in human disease. *Free Radic.Biol.Med.* 37, 1097-1104.

11. Field, A., 2005. Discoverings statistics using SPSS. 2London, SAGE Publications Ltd. ISM Introducing Statistical Methods. Wright, D. B.
12. Gajdos, V., Petit, F.M., Perret, C., Mollet-Boudjemline, A., Colin, P., Capel, L. et al., 2006. Further evidence that the UGT1A1*28 allele is not associated with coronary heart disease: The ECTIM Study. *Clinical Chemistry* 52, 2313-2314.
13. Gullu, H., Erdogan, D., Tok, D., Topcu, S., Caliskan, M., Ulus, T. et al., 2005. High serum bilirubin concentrations preserve coronary flow reserve and coronary microvascular functions. *Arteriosclerosis, Thrombosis and Vascular Biology* 25, 2289-2294.
14. Hong, A.L., Huo, D., Kim, H.J., Niu, Q., Frankenthal, D.L., Cummings, S.A. et al., 2007. UDP-Glucuronosyltransferase 1A1 Gene Polymorphisms and Total Bilirubin Levels in an Ethnically Diverse Cohort of Women. *Drug Metab Dispos.* 35, 1254-1261.
15. Hopkins, P.N., Wu, L.L., Hunt, S.C., James, B.C., Vincent, G.M., Williams, R.R., 1996. Higher serum bilirubin is associated with decreased risk for early familial coronary artery disease. *Arteriosclerosis, Thrombosis and Vascular Biology* 16, 250-255.
16. Hunt, S.C., Kronenberg, F., Eckfeldt, J.H., Hopkins, P.N., Myers, R.H., Heiss, G., 2001. Association of plasma bilirubin with coronary heart disease and segregation of bilirubin as a major gene trait: The NHLBI Family Heart Study. *Atherosclerosis* 154, 747-754.
17. Hunt, S.C., Wu, L.L., Hopkins, P.N., Williams, R.R., 1996. Evidence for a major gene elevating serum bilirubin concentration in Utah pedigrees. *Arteriosclerosis, Thrombosis and Vascular Biology* 16, 912-917.
18. Kronenberg, F., Coon, H., Gutin, A., Abkevich, V., Samuels, M.E., Ballinger, D.G. et al., 2002. A genome scan for loci influencing anti-atherogenic serum bilirubin levels. *Eur.J.Hum.Genet.* 10, 539-546.
19. Levinson, S.S., 1997. Relationship between bilirubin, apolipoprotein B, and coronary artery disease. *Ann.Clin.Lab Sci.* 27, 185-192.
20. Lin, J.P., Cupples, L.A., Wilson, P.W., Heard-Costa, N., O'Donnell, C.J., 2003. Evidence for a gene influencing serum bilirubin on chromosome 2q telomere: a genomewide scan in the Framingham study. *American Journal of Human Genetics* 72, 1029-1034.
21. Lin, J.-P., O'Donnell, C.J., Schwaiger, J.P., Cupples, A., Lingenhel, A., Hunt, S.C. et al., 2006. Association between UGT1A1*28 allele, bilirubin levels, and coronary heart disease in the Framingham Heart Study. *Circulation* 114, 1476-1481.
22. Marin, F., Gonzalez-Conejero, R., Lee, K.W., Corral, J., Roldan, V., Lopez, F. et al., 2005. A pharmacogenetic effect of factor XIII valine 34 leucine polymorphism on fibrinolytic therapy for acute myocardial infarction. *J.Am.Coll.Cardiol.* 45, 25-29.

23. Maruo, Y., D'Addario, C., Mori, A., Iwai, M., Takahashi, H., Sato, H. et al., 2004. Two linked polymorphic mutations (A(TA)₇TAA and T-3279G) of UGT1A1 as the principal cause of Gilbert syndrome. *Human Genetics* 115, 525-526.
24. Maruo, Y., Nishizawa, K., Sato, H., Doida, Y., Shimada, M., 1999. Association of neonatal hyperbilirubinemia with bilirubin UDP-glucuronosyltransferase polymorphism. *Pediatrics* 103, 1224-1227.
25. Morita, T., 2005. Heme oxygenase and atherosclerosis. *Arteriosclerosis, Thrombosis and Vascular Biology* 25, 1786-1795.
26. Neuzil, J., Stocker, R., 1994. Free and albumin-bound bilirubin are efficient co-antioxidants for alpha-tocopherol, inhibiting plasma and low density lipoprotein lipid peroxidation. *Journal of Biological Chemistry* 269, 16712-16719.
27. Novotny, L., Vitek, L., 2003. Inverse relationship between serum bilirubin and atherosclerosis in men: a meta-analysis of published studies. *Exp.Biol.Med.(Maywood.)* 228, 568-571.
28. Öllinger, R., Bilban, M., Erat, A., Froio, A., McDaid, J., Tyagi, S. et al., 2005. Bilirubin: a natural inhibitor of vascular smooth muscle cell proliferation. *Circulation* 112, 1030-1039.
29. Rantner, B., Kollerits, B., Anderwald-Stadler, M., Klein-Weigel, P., Gruber, I., Gehringer, A. et al., 2008. Association between the *UGT1A1* TA-repeat polymorphism and bilirubin concentration in patients with intermittent claudication: results from the CAVASIC Study. *Clinical Chemistry* 54, 851-857.
30. Schoenborn, V., Heid, I.M., Vollmert, C., Lingenhel, A., Adams, T.D., Hopkins, P.N. et al., 2006. The *ATGL* gene is associated with free fatty acids, triglycerides and type 2 diabetes. *Diabetes* 55, 1270-1275.
31. Schwertner, H.A., Fischer, J.R., Jr., 2000. Comparison of various lipid, lipoprotein, and bilirubin combinations as risk factors for predicting coronary artery disease. *Atherosclerosis* 150, 381-387.
32. Schwertner, H.A., Jackson, W.G., Tolan, G., 1994. Association of low serum concentration of bilirubin with increased risk of coronary artery disease. *Clinical Chemistry* 40, 18-23.
33. Schwertner, H.A., Vitek, L., 2008. Gilbert syndrome, UGT1A1*28 allele, and cardiovascular disease risk: possible protective effects and therapeutic applications of bilirubin. *Atherosclerosis* 198, 1-11.
34. Stocker, R., Glazer, A.N., Ames, B.N., 1987. Antioxidant activity of albumin-bound bilirubin. *Proceedings of the National Academy of Sciences of the United States of America* 84, 5918-5922.

35. The Wellcome Trust Case Control Consortium, 2007. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 447, 661-678.
36. Troughton, J.A., Woodside, J.V., Young, I.S., Arveiler, D., Amouyel, P., Ferrieres, J. et al., 2007. Bilirubin and coronary heart disease risk in the Prospective Epidemiological Study of Myocardial Infarction (PRIME). *Eur.J.Cardiovasc.Prev.Rehabil.* 14, 79-84.
37. Vitek, L., Schwertner, H.A., 2007. The heme catabolic pathway and its protective effects on oxidative stress-mediated diseases. *Adv.Clin Chem.* 43, 1-57.

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Table 1: Baseline clinical and laboratory data of male and female patients with coronary artery disease and the respective controls

| | Males | | Females | |
|--------------------------------------|---|---|---|---|
| | Controls (n=397) | Patients (n=365) | Controls (n=222) | Patients (n=112) |
| Age (years) | 54±9 | 54±7 | 57±7 | 59±8 |
| Body mass index (kg/m ²) | 28.6±4.5 | 28.7±4.6 | 27.5±5.9 | 29.0±6.2 ^a |
| Serum creatinine (mg/dl) | 1.04±0.31 | 1.13±0.66 ^d | 0.84±0.21 | 0.93±0.23 ^c |
| Serum albumin (g/dl) | 4.33±0.27 | 4.22±0.34 ^d | 4.23±0.27 | 4.08±0.33 ^d |
| Serum glucose (mg/dl) | 95±21 | 101±46 ^a | 90±16 | 102±52 ^a |
| Serum bilirubin (mg/dl) | 0.76±0.41 [0.50, 0.70, 0.90] | 0.62±0.36^e [0.40, 0.56, 0.77] | 0.55±0.23 [0.40, 0.50, 0.60] | 0.42±0.29^f [0.21, 0.34, 0.50] |
| Total cholesterol, (mg/dl) | 184±33 | 215±47 ^d | 198±34 | 229±46 ^d |
| LDL cholesterol, (mg/dl) | 106±29 | 136±42 ^d | 108±29 | 138±35 ^d |
| HDL cholesterol, (mg/dl) | 43.2±11.8 | 38.1±10.2 ^d | 58.2±15.2 | 47.5±13.4 ^d |
| Triglycerides, (mg/dl) | 167±119 [101, 142, 201] | 204±138 ^d [117, 164, 245] | 158±87 [100, 139, 191] | 219±162 ^d [129, 181, 282] |
| Smoking (Non/Former/Current), % | 72 / 24 / 4 | 39 / 43 / 18 ^d | 83 / 15 / 2 | 56 / 27 / 17 ^d |

Data are mean ± SD and [25th, 50th (=median), 75th percentile] where appropriate.

^a p<0.05; ^b p<0.01; ^c p<0.005; ^d p<0.001, ^e p=1.2x10⁻¹⁰, ^f p=1.9x10⁻⁹ for comparison between patients and respective controls

Table 2: Age-adjusted *UGT1A1* genotype-specific bilirubin levels of controls and patients with coronary artery disease stratified by gender

| Genotype | Control group | | | | CAD group | | | | P value ^c CAD vs. controls |
|------------------------------------|---------------|---|-----------------------|-----------------------|------------------------------|---|-----------------------|-----------------------|---|
| | n (%) | Bilirubin (mg/dl) ^a mean (95% CI) | P value ^b | | n (%) | Bilirubin (mg/dl) ^a mean (95% CI) | P value ^b | | |
| | | | additive | recessive | | | additive | recessive | |
| -53(TA-repeat) polymorphism | | | | | | | | | |
| Men | | | | | | | | | |
| 6/6 | 193(49.7) | 0.64(0.61-0.68) | | | 168(46.8) | 0.50(0.46-0.54) | | | 1.1x10 ⁻⁷ |
| 6/7 | 152(39.2) | 0.70(0.66-0.74) | 4.2x10 ⁻¹⁸ | 5.9x10 ⁻²⁶ | 153(42.6) | 0.59(0.55-0.64) | 1.4x10 ⁻¹⁴ | 3.4x10 ⁻¹⁶ | 0.0004 |
| 7/7 | 43(11.1) | 1.24(1.14-1.35) | | | 38(10.6) | 1.01(0.90-1.13) | | | 0.06 |
| Minor allele frequency=30.7% | | | | | Minor allele frequency=31.9% | | | | |
| Women | | | | | | | | | |
| 6/6 | 88(40.6) | 0.52(0.48-0.57) | | | 50(47.6) | 0.33(0.27-0.39) | | | 0.000001 |
| 6/7 | 103(47.4) | 0.53(0.49-0.57) | 0.20 | 0.09 | 44(41.9) | 0.41(0.35-0.48) | 0.00002 | 0.00001 | 0.00035 |
| 7/7 | 26(12.0) | 0.60(0.52-0.68) | | | 11(10.5) | 0.73(0.58-0.90) | | | 0.15 |
| Minor allele frequency=31.9% | | | | | Minor allele frequency=31.4% | | | | |
| SNP T-3279G | | | | | | | | | |
| Men | | | | | | | | | |
| T/T | 136(35.8) | 0.63(0.58-0.68) | | | 106(31.7) | 0.50(0.44-0.55) | | | 0.00003 |
| T/G | 165(43.4) | 0.69(0.65-0.74) | 2.2x10 ⁻¹¹ | 3.0x10 ⁻¹² | 165(49.4) | 0.57(0.53-0.62) | 1.3x10 ⁻⁸ | 1.3x10 ⁻⁸ | 0.00003 |
| G/G | 79(20.8) | 0.95(0.87-1.02) | | | 63(18.9) | 0.80(0.72-0.89) | | | 0.07 |
| Minor allele frequency=42.5% | | | | | Minor allele frequency=43.6% | | | | |
| Women | | | | | | | | | |
| T/T | 54(25.6) | 0.54(0.48-0.59) | | | 27(27.0) | 0.34(0.26-0.42) | | | 0.0005 |
| T/G | 107(50.7) | 0.52(0.48-0.56) | 0.83 | 0.58 | 51(51.0) | 0.36(0.30-0.42) | 0.0005 | 0.00004 | 7.0x10 ⁻⁷ |
| G/G | 50(23.7) | 0.54(0.49-0.60) | | | 22(22.0) | 0.60(0.50-0.72) | | | 0.32 |
| Minor allele frequency=49.1% | | | | | Minor allele frequency=47.5% | | | | |

^a The mean bilirubin levels are the retransformed mean bilirubin levels computed on the ln(bilirubin+1) scale.

^b p values from linear regression adjusted for age to compare bilirubin levels between the genotype groups within controls or CAD cases. The additive genetic model calculates the p values for the changes of mean bilirubin levels per copy of the minor allele. The recessive genetic model calculates the p values for the changes of mean bilirubin levels of subjects with two copies of the minor allele versus subjects with only one or no copy of the minor allele.

^c p values from linear regression adjusted for age to compare bilirubin levels between controls and CAD cases with the same genotype group.

Table 3: Percentage of variation of bilirubin concentrations explained by classical factors and the -53(TA-repeat) and the T-3279G polymorphism (assuming a recessive genetic model). Values given are the r^2 values (as percentage) from linear regression with various adjustments.

| | r^2 (%) full model ^a | r^2 (%) submodel ^b | r^2 (%) explained by polymorphisms ^c |
|---|--------------------------------------|------------------------------------|--|
| -53(TA-repeat) polymorphism | | | |
| Cases | 24.1 | 8.7 | 15.4 |
| Controls | 23.6 | 10.5 | 13.1 |
| SNP T-3279G | | | |
| Cases | 17.9 | 8.7 | 9.2 |
| Controls | 17.0 | 10.5 | 6.5 |
| -53(TA-repeat) polymorphism and SNP T-3279G combined | | | |
| Cases | 24.1 | 8.7 | 15.4 |
| Controls | 24.1 | 10.5 | 13.6 |

^a adjusted for age, gender, smoking status, and either of the two polymorphisms or the two polymorphisms combined;

^b same as ^a but without adjustment for any polymorphism;

^c The variation explained by the polymorphisms was calculated by the difference of r^2 between a full model ^a and the submodel ^b.

Table 4: Logistic regression analysis adjusted for age and stratified for gender investigating the association of bilirubin concentrations and the -53(TA-repeat) and the T-3279G polymorphisms (genotypes) on the change of odds for coronary artery disease (CAD). Values given are the odds ratio (OR), 95% confidence intervals (CI), and the respective p-value testing for deviance of the OR from unity.

| | Women | | Men | |
|--|-----------------|----------------------|-----------------|----------------------|
| | OR (95% CI) | P value | OR (95% CI) | P value |
| Bilirubin (increment 0.1mg/dl) | | | | |
| Model 1 ^a | 0.77(0.68-0.87) | 3.2x10 ⁻⁵ | 0.90(0.86-0.94) | 2.6x10 ⁻⁶ |
| Model 2 ^b | 0.72(0.61-0.85) | 0.0002 | 0.93(0.88-0.98) | 0.005 |
| Model 3 ^c | 0.79(0.66-0.94) | 0.009 | 0.94(0.89-0.99) | 0.018 |
| -53(TA-repeat) polymorphism | | | | |
| Additive model (per copy of 7 repeats) | 0.85(0.60-1.21) | 0.37 | 1.06(0.86-1.31) | 0.59 |
| Recessive model (7/7 vs. 6/6+6/7) | 0.93(0.45-1.93) | 0.84 | 0.97(0.61-1.54) | 0.90 |
| SNP T-3279G | | | | |
| Additive model (per copy of G) | 0.95(0.68-1.33) | 0.76 | 1.04(0.85-1.27) | 0.71 |
| Recessive model (G/G vs. T/T+T/G) | 0.92(0.52-1.62) | 0.78 | 0.88(0.61-1.28) | 0.51 |

^a adjusted for age

^b adjusted for age, LDL cholesterol, HDL cholesterol, BMI, smoking, serum creatinine

^c as in ^b, but additionally adjusted for glucose and albumin