Anti-Genotoxic Potential of Bilirubin In Vivo: Damage to DNA in Hyperbilirubinemic Human and Animal Models

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Abstract

The bile pigment bilirubin is a known antioxidant and is associated with protection from cancer and cardiovascular disease (CVD) when present in too strong concentrations. Unconjugated bilirubin (UCB) might also possess anti-genotoxic potential by preventing oxidative damage to DNA. Moderately elevated bilirubin levels are found in individuals with Gilbert syndrome and more severe in the hyperbilirubinemic Gunn rat model. This study was therefore aimed to assess the levels of oxidative damage to DNA in Gilbert syndrome subjects and Gunn rats compared to matched controls. Seventy-six individuals (age- and sex-matched) were allocated into Gilbert syndrome (UCB > 17.1 µmol/L; n = 38) or control groups (UCB < 17.1 µmol/L; n = 38). In addition, 40 Gunn rats were used to support the results of the human trial. Single-cell gel electrophoresis (SCGE) assay measuring standard conditions (strand breaks, apurinic/apyrimidinic sites) and formamidopyrimidine glycosylase (FPG)-sensitive sites was conducted in human peripheral blood mononuclear cells (PBMC) and rat PBMCs, colon, and hepatocytes. Furthermore, urinary 8-oxo-2′-deoxyguanosine (8oxoGuo, DNA oxidation) and 8-oxoguanosine (8oxoG, RNA oxidation) were measured in humans. The Gilbert syndrome and Gunn rat groups had significantly higher UCB levels (P < 0.001) than the corresponding controls. No further differences in damage to DNA or RNA were detected between the two groups, except higher strand breaks (PBMCs) in Gunn rats when compared with controls. However, when demographic effects were analyzed, lower 8oxoGuo concentrations were detected in the human group with a BMI ≥ 25 kg/m² (1.70 ± 0.67 vs. 1.38 ± 0.43 nmol/mmol creatinine, P < 0.05), although this group showed lower UCB levels than normal weight subjects. This study suggests that the disease preventative effect of UCB is unrelated to DNA oxidation/strand breaks in human and animal models of hyperbilirubinaemia.

Cancer Prev Res; 6(10); 1056–63. © 2013 AACR.

Introduction

Reactive oxygen species (ROS) from endogenous and exogenous sources can cause severe oxidative damage to organic macromolecules such as lipids, proteins, and DNA (1). Lesions in the double helix of DNA lead to genomic instability and replication failure if they remain unrepaired. Subsequently, it might result in increased mutation processes and carcinogenesis (2).

Bilirubin is a physiologically important antioxidant and, therefore, might assist in neutralizing ROS and preventing oxidative damage (3, 4). The heme-derived bile pigment is moderately elevated in a benign condition known as Gilbert syndrome, affecting 3% to 13% of the general population (~12.4% men, ~4.8% women; ref. 5), which is caused by additional TA repeats in the gene promoter for bilirubin UDP-glucuronosyl transferase (UGT1A1), specifically UGT1A1*28 polymorphism. This promoter mutation substantially decreases transcription of UGT1A1, resulting in reduced capacity of the liver to conjugate bilirubin with glucuronic acid and its retention in the systemic circulation (6).

Importantly, epidemiologic evidence suggests a preventive role for bilirubin in disease development, specifically in regard to cardiovascular disease and cancer (7, 8). These associations are also reported in individuals with Gilbert syndrome (9, 10). Several findings suggest preventive effects...
of unconjugated bilirubin (UCB) on cancer development, showing that the prevalence and incidence of lung and colon cancer is reduced with increasing circulating UCB concentrations (7, 11). Recently, a possible protective role of the UGT1A1*28 allele on colorectal cancer (CRC) was detected and patients with CRCs had lower serum bilirubin levels than controls (10). In vitro anti-oxidative and anti-genotoxic properties of bilirubin were shown in recent reports (12–14). Therefore, it is hypothesized that subjects with elevated bilirubin levels are more resistant to oxidative stress–related diseases because Gilbert syndrome individuals possess reduced levels of oxidative stress (15, 16).

The single-cell gel electrophoresis (SCGE)/comet assay (17) and measurements of modified nucleoside 8-oxo-2′-deoxyguanosine (8oxodGuo) provide information on oxidative damage to DNA and nucleoside 8-oxo-guanosine (8oxoGuo) measures oxidative damage to RNA (18).

So far, only one study (19) has used the SCGE assay to assess the relationship between DNA damage in human PBMCs and the UGT1A1*28 genotype. This study was conducted on a small sample size (n = 28) and UCB levels of individual subjects were not presented. Another very recent study reported lower 8oxodGuo levels in male Gilbert syndrome subjects only (15).

In this study, it was hypothesized that moderately elevated bilirubin levels reduce oxidative modification of DNA and RNA and protect hyperbilirubinemic individuals against oxidative stress. Single- and double-strand DNA breaks and FPG-sensitive sites (oxidized purines) in isolated human peripheral blood mononucleated cells (PBMCs) which reflect damage to DNA were assessed by the SCGE assay (% DNA in tail). PBMCs were additionally challenged with hydrogen peroxide (H2O2) to assess their ROS sensitivity. Furthermore, 8oxodGuo and 8oxoGuo were measured in human urine. In addition, a variety of antioxidants and vitamins were measured in human samples to assess their possible influence on the damage to DNA. For further investigation of DNA modulations in different tissues, an animal study was conducted comparing normo- versus hyperbilirubinemic rats. The SCGE assay (strand breaks, FPG-sensitive sites) was conducted in rat PBMCs, colonocytes, and hepatocytes and whole-body γ-irradiation was conducted to challenge the animals in vivo.

Materials and Methods

Human study: design and subjects
Within this study, 104 subjects were recruited from the general population. Seventy-six subjects met the inclusion criteria as they possessed normal liver function, absence of disease, and were aged between 20 and 80 years. Reticulocytes, γ-glutamyl transferase (γ-GT), alanine transferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase, hemoglobin, and hematocrit were measured at the screening examination. Subjects with liver, heart or kidney disease, hemolysis, diabetes, cholelithiasis, organ transplants, history of cardiovascular disease (CVD), cancer, smoking (>1 cig/d), alcohol consumption (>7 standard drinks/wk), excessive physical activity (>10 h/wk), and any medication that might affect liver metabolism and vitamin supplementation (4 weeks prior the first blood sampling) were excluded. Allocation to the Gilbert syndrome group, as usually performed (20), was determined by a fasting serum UCB concentration of >17.1 µmol/L measured by high-performance liquid chromatography (HPLC; see below). Subjects were age- and sex-matched in each group (total 76 subjects; 2 groups of each 28 men and 10 women). The study was approved by the Ethical Committee of the Medical University of Vienna and the General Hospital of Vienna (# 274/2010) and conducted according to the Declaration of Helsinki.

Sample preparation
After 24-hour of fasting (400 kcal food restriction), blood samples were collected into lithium heparin and serum vacutainers (Vacuette). Samples were stored on ice in the dark until further analysis. Blood collection tubes were centrifuged (10 minutes, 3,000 rpm, 4°C), and serum or plasma was aliquoted, used fresh or stored at −80°C for further analysis. Erythrocytes from lithium heparin vacutainers were washed 3 times with isotonic phosphate buffer, aliquoted, and stored at −80°C. Spot urine samples were collected, aliquoted, and stored at −20°C.

Determination of 8oxodGuo, 8oxoGuo, and creatinine
8oxodGuo and 8oxoGuo were determined in urine at the Laboratory of Clinical Pharmacology, Rigshospitalet, Copenhagen. In brief, a chromatographic separation was conducted on an Acquity UPLC system, using an Acquity UPLC BEH Shield RP18 column (1.7 μm, 2.1 × 100 mm²) and a gradient of A: 2.5 mmol/L ammonium acetate (pH 5) and B: acetonitrile. The MS/MS detection was conducted on an API 3000 triple quadrupole mass spectrometer using electrospray ionization operated in the positive mode (18). 15N5-8oxoGuo and 15N5-8oxodGuo were applied as internal standards. Two specific product ions were measured from each analyte to ensure correct identification and quantification. 8oxodGuo and 8oxoGuo were normalized against urinary creatinine concentration, which were measured using routine diagnostic tests on Olympus 5400 (Beckman Coulter) at the General Hospital of Vienna.

Blood biochemistry
Liver function enzymes γ-GT, AST, ALT, LDH, and ALP were analyzed using routine diagnostic tests on Olympus 5400 clinical chemistry analyzer (Beckman Coulter) and measured on the day of the first blood sampling.

Determination of vitamins, antioxidants, and homocysteine
Plasma concentrations of β-carotene were determined by reverse-phase HPLC (21). One milliliter of plasma was mixed with 1 mL ethanol, 100 μL internal standard (dihydrophyllochinone), 5 mL hexane, mixed thoroughly, and centrifuged for 3 minutes at 3,000 rpm. For the analysis of
carotenoids, 500 μL of the hexane phase was dried under nitrogen stream and dissolved in mobile phase (72% acetone, 10% methanol, and 18% dichloromethane). The column (LiChrospher 100, RP-18, 5 μm, 250 × 4 mm², Merck) was thermostatically controlled at 20°C and a UV detector at 450 nm was used. Vitamin C (22) and glutathione (23) were determined by spectrophotometry using a UV/VIS spectrometer (Hitachi). To ensure quality control, a control plasma sample was run throughout the study. Coefficients of variation (CV) for vitamins and antioxidants were between 2% and 8%.

Vitamin B12 was measured in plasma and folic acid in erythrocytes using radioimmunoassays (MP Biomedicals). Plasma homocysteine was determined using HPLC with a fluorescence detector (emission wavelength: 515 nm; excitation wavelength: 385 nm) on a RP LiChrosphere column (5 μm, 125 × 4 mm²; Merck, Hitachi, LaChrom). Potassium hydrogen phosphate buffer including 4% acetoneitrile was used as mobile phase (24).

**Animal study: design and treatment of animals**

Twenty hyperbilirubinemic Gunn rats (homozygous for a mutation in \( \text{UGT1A1, jj} \)) and 20 respective controls (non-mobilirubinemic Wistar rats, heterozygous for a mutation in \( \text{UGT1A1, jj} \)) were obtained from Charles University in Prague (Czech Republic) and acclimatized in the breeding facility of the Medical University of Vienna (Himberg, Austria) 1 week before experiments. The animals were housed under standard conditions (24°C, humidity 50% ± 20%, 12-hour light:dark cycle) and fed with a standard diet (sniift R/M-H Extrudat, sniff Spezialdiäten GmbH, Germany) and ad libitum access to fresh water. All experiments were carried out with 7- to 8-week-old animals (total number 40). The study was approved by the committee of animal experiments of the Austrian Federal Ministry of Science and Research (BMF-66.006/0008-II/3b/2011).

The animals were randomly allocated into a no-treatment group (9 jj and 9 jj rats) or a treatment group (11 jj and 11 jj rats). The treatment group was \( \gamma \)-irradiated (\( ^{60} \text{Co} \) source) with a total dose of 10.12 Gy, which was chosen from results of our pre-experiments.

**Sample preparation**

Animals were sacrificed immediately after irradiation by decapitation after \( \text{CO}_2 \) asphyxiation. Immediately blood, colon, and liver were immediately removed. The colon was washed 4 times with PBS, and cells were collected by light scraping and transferred into cold (4°C) PBS solution. One gram of liver was transferred into cold buffer (pH 7.5) and homogenized (Potter-Elvehjem), centrifuged (800 × g, 4°C, 10 minutes), and the supernatant was used for SCEG assay (25). Blood was collected into sodium-heparinized tubes (Ebewe) and immediately transferred into Ficoll-containing tubes (Greiner Bio-One) for PBMC isolation. Plasma was obtained after centrifugation, aliquoted, and stored at −80°C until further analysis. Isolated PBMCs were washed 2 times with cold PBS and further treated at 4°C.

**Determination of biomarkers in human and animal samples**

**Determination of UCB.** UCB was determined in serum (humans) and heparinized plasma (animals) immediately after centrifugation or within 1 week, if replicates were required (25). HPLC (Merck, Hitachi, LaChrom) equipped with a photo diode array detector (PDA, Shimadzu) was used to measure UCB eluted from a Fortis C18 HPLC column (4.6 × 150 mm, 3 μm) with a Phenomenex C18 HPLC guard column (4.0 × 3.0 mm; refs. 20, 26). An isocratic mobile phase perfused the column and contained 0.1 mol/L di-\( n \)-octylamine in methanol-water (95:5; v/v) and glacial acetic acid. UCB was extracted from serum by mixing 40 μL serum with 160 μL mobile phase. After centrifugation, 50 μL of the supernatant was injected at a flow rate of 1 ml/min. Retention time of the IXa UCB peak was 10.9 minutes. UCB acted as an external control and possessed an isomeric purity of >99% (for 3.3% III\( \alpha \), 92.8% IX\( \alpha \), and 3.9% XIII\( \alpha \) isomers, respectively; 450 nm; Frontier Scientific).

**Isolation of PBMCs**

PBMCs were isolated from lithium-heparinized blood via Ficoll separation tubes (Greiner bio one). Then, the cells were separated by centrifugation (3,100 rpm, 25 minutes, room temperature) according to the instructions and washed twice with cold PBS.

**SCEG assay**

The SCEG assay was applied in PBMCs, liver, and colonocytes (17, 27). Single- and double-strand DNA breaks and FPG-sensitive sites were measured in all human and rodent cells. DNA damage was induced in human PBMCs by \( \text{H}_2\text{O}_2 \) (100 μmol/L, all, \( \text{ex vivo} \)) and in rodents (22 rats of 40, \( \text{in vivo} \)) by \( \gamma \)-irradiation (10.12 Gy). Thirty micro-liters (\( \sim \)1 ×10⁶ cells/ml) was mixed with 140 μL of 1% low melting point agarose in PBS at 37°C and applied onto precoated (1% normal melting point agarose in distilled water) slides. Duplicates (strands breaks, \( \text{H}_2\text{O}_2 \) or tripli-cates (FPG) were conducted. The \( \text{H}_2\text{O}_2 \) treatment was conducted for 5 minutes at 4°C. Then, all slides were placed into lysis buffer (pH 10) for a minimum of 1 hour.

For enzyme treatment, 50 μL enzyme-containing buffer (FPG) or enzyme buffer only was pipetted on the spot and incubated for exactly 30 minutes. The DNA unwinding phase was conducted for 20 minutes. In the same solution, the electrophoresis was conducted for 30 minutes and 25 V (300 mA) at pH > 13. Evaluation was performed after ethidium bromide staining (20 μg/mL). Two replicates (50 cells per slide were evaluated) for each sample were analyzed, and the mean (% DNA in tail) was measured on a fluorescent microscope (Zeiss) using Komet 5.5 software (Kineting Imaging).

**Statistical analysis**

All statistical tests were completed using SPSS (IBM statistics, Version 17.0). Normal distribution within the data set was tested using the Kolmogorov–Smirnov test. To determine differences between 2 groups, an independent...
sample t test (parametric data) or Mann–Whitney U test (nonparametric data) was conducted. Dependent on the homogeneity of variances and normality of data within groups, one-way ANOVA (parametric data) or Kruskal–Wallis H Test (nonparametric data) was used for multiple group comparisons. Pearson coefficient (parametric data) or Spearman ρ correlation (non-parametric data) tested the bivariate relationships between independent and dependent variables. Data are expressed as mean ± SD and P < 0.05 was considered significant for differences.

Results

Results of the human study

The Gilbert syndrome group had a significantly lower BMI (P = 0.025) and higher UCB concentrations (P < 0.001) than the age- and sex-matched controls. The concentrations of liver enzymes between both groups did not differ (Table 1).

No significant difference between Gilbert syndrome and control group regarding levels of damage to DNA (standard conditions, H2O2, FPG-sensitive sites, and 8oxodGuo and RNA (8oxoGuo) was found. Similarly, antioxidant variables. Data are expressed as mean ± SD and P < 0.05 was considered significant for differences.

Table 1. Demographic features, biochemical markers of oxidative stress, and DNA damage and vitamins of controls and individuals with Gilbert syndrome

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls</th>
<th>Gilbert syndrome</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>28/10</td>
<td>28/10</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>31.9 (11.2)</td>
<td>32.3 (11.8)</td>
<td>0.881</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.4 (3.22)</td>
<td>22.8 (2.85)</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>UCB, μmol/L</td>
<td>10.3 (3.31)</td>
<td>12.0 (13.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>γ-GT, U/L</td>
<td>19.5 (7.08)</td>
<td>22.2 (14.4)</td>
<td>0.300</td>
</tr>
<tr>
<td>AST, U/L</td>
<td>24.3 (7.22)</td>
<td>26.7 (7.35)</td>
<td>0.165</td>
</tr>
<tr>
<td>ALT, U/L</td>
<td>21.7 (8.27)</td>
<td>25.5 (14.4)</td>
<td>0.166</td>
</tr>
<tr>
<td>LDH, U/L</td>
<td>157 (23.7)</td>
<td>163 (26.6)</td>
<td>0.315</td>
</tr>
<tr>
<td>ALP, U/L</td>
<td>64.0 (14.9)</td>
<td>70.1 (17.3)</td>
<td>0.105</td>
</tr>
<tr>
<td>Standard conditions²b</td>
<td>5.08 (1.19)</td>
<td>5.13 (1.34)</td>
<td>0.862</td>
</tr>
<tr>
<td>H2O2-sensitive sites²</td>
<td>18.6 (4.49)</td>
<td>19.5 (4.80)</td>
<td>0.608</td>
</tr>
<tr>
<td>FPG-sensitive sites²</td>
<td>4.97 (2.07)</td>
<td>4.62 (2.58)</td>
<td>0.608</td>
</tr>
<tr>
<td>8oxodG²</td>
<td>1.86 (0.52)</td>
<td>1.86 (0.64)</td>
<td>0.466</td>
</tr>
<tr>
<td>8oxoGuo²</td>
<td>1.59 (0.54)</td>
<td>1.62 (0.70)</td>
<td>0.848</td>
</tr>
<tr>
<td>Vitamin C, μmol/L</td>
<td>73.2 (12.5)</td>
<td>71.5 (13.1)</td>
<td>0.570</td>
</tr>
<tr>
<td>β-carotene, μmol/L</td>
<td>0.59 (0.41)</td>
<td>0.61 (0.44)</td>
<td>0.794</td>
</tr>
<tr>
<td>Glutathione, mg/dL</td>
<td>74.2 (9.03)</td>
<td>72.0 (11.0)</td>
<td>0.329</td>
</tr>
<tr>
<td>Folic acid, ng/mL</td>
<td>142 (120)</td>
<td>142 (116)</td>
<td>0.608</td>
</tr>
<tr>
<td>Vitamin B12, ng/L</td>
<td>280 (125)</td>
<td>283 (121)</td>
<td>0.900</td>
</tr>
</tbody>
</table>

NOTE: Values as mean (SD).

²% DNA in tail.

³Strand breaks and apurinic sites.

⁴nmol/mmol creatinine.

As DNA damage might be influenced by age, body mass index (BMI), and sex, more specific analyses were conducted by dividing the human cohort into groups. Two age subgroups (age group 1: <30 years, age group 2: ≥30 years; with a range from 30 to 72 years) were built and the influence of sex and BMI (<25 kg/m², ≥25 kg/m²) were taken into consideration (Table 2). The older age group (≥30 years) had a significantly higher BMI (P < 0.05) and tended to show greater oxidative stress (8oxoGuo, P = 0.067). Females had greater levels of 8oxoGuo (P < 0.05) than in males, although, a trend to lower levels of strand breaks detected by the comet assay (P = 0.060) was found. The group with lower BMI had significantly higher UCB concentrations and was younger than the group with a BMI ≥25 kg/m². Furthermore, significantly lower levels of 8oxodGuo were found in the higher BMI group, and a trend to increased strand breaks (P = 0.064) was detected among this group compared to individuals with a lower BMI.

UCB correlated significantly with BMI (r = −0.292, P < 0.05). Urinary 8oxodGuo was negatively correlated to BMI (r = −0.251, P < 0.05) and total glutathione (r = −0.259, P < 0.05) and positively to 8oxoGuo (r = 0.519, P < 0.05). Moreover, 8oxoGuo, was positively correlated to age (r = 0.386, P < 0.05) and negatively to vitamin B12 (r = −0.265, P < 0.05). In addition, vitamin B12 was negatively correlated to strand breaks (r = −0.441, P < 0.05) and FPG-sensitive sites (r = −0.372, P < 0.05).

Results of the animal study

The findings of the animal trial are summarized in Table 3. Hyperbilirubinemic Gunn rats had significantly greater UCB concentrations than Wistar rats (P < 0.001). PBMCs of the treatment groups (with irradiation) showed significantly greater damage to DNA than the nonirradiated groups (P < 0.001). Greater numbers of strand breaks/apurinic sites were also found in treated colonocytes and hepatocytes (P < 0.001). Interestingly, in the absence of irradiation hyperbilirubinemic rats had significantly greater strand breaks/apurinic sites in PBMCs than the normobilirubinemic rats (P < 0.05).

Discussion

This study aimed to investigate a DNA protective effect of moderately elevated bilirubin in hyperbilirubinemic humans and rodents. Bilirubin is a known antioxidant (12) and protects against oxidative damage to DNA (15) which might be due to its ROS scavenging potential (28). Surprisingly, greater strand breaks in PBMCs were found in the hyperbilirubinemic rats in the absence of irradiation (Table 3). This could either be due to higher DNA damage itself or might indicate efficient DNA repair (29). The UCB concentrations in some of the Gunn animals were approximately 3 times higher than in Gilbert syndrome and it is well-known that severely elevated UCB concentrations induce toxic effects (30). Simultaneous administration of toxic UCB doses (50 mg/kg) and whole-body γ-irradiation of mice led to the greatest induction of immunotoxic effects. A reduced spleen weight, lower viability...
Table 2. UCB concentration, demographic features, and DNA damage of controls and individuals with Gilbert syndrome subdivided in groups of age, gender, and BMI

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Age &lt;30 y  (n = 43)</th>
<th>Age ≥30 y (n = 33)</th>
<th>Gender Male (n = 56)</th>
<th>Gender Female (n = 20)</th>
<th>BMI &lt;25 kg/m² (n = 53)</th>
<th>BMI ≥25 kg/m² (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCB, μmol/L</td>
<td>19.9 (13.2)</td>
<td>22.7 (16.5)</td>
<td>22.4 (15.5)</td>
<td>17.5 (11.6)</td>
<td>23.6 (16.1)</td>
<td>15.3 (8.55)</td>
</tr>
<tr>
<td>Age, y</td>
<td>24.3 (2.50)</td>
<td>42.2 (10.5)d</td>
<td>31.5 (11.4)</td>
<td>33.9 (11.7)</td>
<td>29.7 (9.86)</td>
<td>37.7 (13.0)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.2 (2.83)</td>
<td>24.8 (3.11)d</td>
<td>23.8 (2.89)</td>
<td>22.9 (3.71)</td>
<td>21.9 (1.75)</td>
<td>27.4 (1.91)</td>
</tr>
<tr>
<td>Standard conditionsa,b</td>
<td>5.06 (1.31)</td>
<td>5.17 (1.27)</td>
<td>5.27 (1.21)</td>
<td>4.65 (1.31)f</td>
<td>4.93 (1.26)</td>
<td>5.51 (1.16)</td>
</tr>
<tr>
<td>H₂O₂-sensitive sitesa</td>
<td>19.6 (4.68)</td>
<td>18.3 (4.55)</td>
<td>19.1 (4.78)</td>
<td>19.0 (4.33)</td>
<td>19.5 (4.77)</td>
<td>18.1 (4.26)</td>
</tr>
<tr>
<td>FPG-sensitive sitesa</td>
<td>5.13 (3.08)</td>
<td>4.38 (2.43)</td>
<td>4.98 (2.96)</td>
<td>4.31 (2.40)</td>
<td>4.71 (2.87)</td>
<td>5.02 (2.77)</td>
</tr>
<tr>
<td>8oxodGc</td>
<td>1.66 (0.60)</td>
<td>1.53 (0.66)</td>
<td>1.58 (0.65)</td>
<td>1.66 (0.56)</td>
<td>1.70 (0.67)</td>
<td>1.38 (0.43)</td>
</tr>
<tr>
<td>8oxoGuo</td>
<td>1.75 (0.49)</td>
<td>2.00 (0.66)a</td>
<td>1.78 (0.58)</td>
<td>2.10 (0.53)a</td>
<td>1.82 (0.55)</td>
<td>1.94 (0.64)</td>
</tr>
</tbody>
</table>

NOTE: Values as mean (SD).

a% DNA in tail.
bStrand breaks and apurinic sites.
cnmol/mmol creatinine.
dP < 0.05 from age < 30 years.
eP = 0.067 from age < 30 years.
fP = 0.060 from males.
gP < 0.05 from males.
hP < 0.05 from BMI < 25 kg/m².
iP = 0.064 from BMI < 25 kg/m².

Table 3. Unconjugated bilirubin, body mass, and DNA damage in PBMCs, colonocytes, and hepatocytes of matched Wistar and Gunn rats

<table>
<thead>
<tr>
<th>Parametersa</th>
<th>Wistar (n = 20)</th>
<th>Gunn (n = 20)</th>
<th>P ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>4/5</td>
<td>4/5</td>
<td>0.699</td>
</tr>
<tr>
<td>UCB, μmol/L</td>
<td>0.58 (0.20)</td>
<td>104 (28)c</td>
<td>0.001</td>
</tr>
<tr>
<td>UCB min/max, μmol/L</td>
<td>0.40/0.92</td>
<td>71.3/167</td>
<td>0.001</td>
</tr>
<tr>
<td>Body mass, g</td>
<td>206 (32)</td>
<td>186 (44)</td>
<td>0.699</td>
</tr>
<tr>
<td>PBMCs</td>
<td>209 (35)</td>
<td>200 (65)</td>
<td>0.699</td>
</tr>
<tr>
<td>Standard conditionsb</td>
<td>6.86 (1.16)d</td>
<td>9.20 (2.22)d,e</td>
<td>0.001</td>
</tr>
<tr>
<td>FPG-sensitive sites</td>
<td>28.4 (6.32)d</td>
<td>33.6 (7.74)d</td>
<td>0.001</td>
</tr>
<tr>
<td>Colonocytes</td>
<td>6.00 (0.19)</td>
<td>36.6 (5.22)</td>
<td>0.001</td>
</tr>
<tr>
<td>Standard conditionsb</td>
<td>16.9 (6.00)d</td>
<td>13.0 (2.88)d</td>
<td>0.001</td>
</tr>
<tr>
<td>FPG-sensitive sites</td>
<td>18.4 (8.16)</td>
<td>20.0 (6.68)</td>
<td>0.508</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>8.29 (1.41)d</td>
<td>8.76 (1.78)d</td>
<td>0.001</td>
</tr>
<tr>
<td>Standard conditionsb</td>
<td>18.7 (6.17)</td>
<td>15.7 (3.41)d</td>
<td>0.05</td>
</tr>
</tbody>
</table>

NOTE: Values as mean (SD).
aUnit of DNA damage parameters: % DNA in tail.
bStrand breaks and apurinic sites.
Least significant difference (LSD) or Games-Howell post hoc tests for differences from Wistar groups.
LSD or Games-Howell post hoc tests for differences from treatment groups.
t test difference from Wistar without treatment.
10.12 Gy radiation dose.
of splenocytes, and decreased counts of lymphocyte subsets compared with untreated mice were reported; therefore, it was suggested that UCB has radiomodifying effects in vivo. These conditions led also to apoptosis in mouse lymphocytes and bone marrow compared with no treatment or one treatment (UCB or irradiation) group (31).

No further differences in the levels of oxidatively damaged DNA between hyperbilirubinemic and normobilirubinemic groups (human and animal) were observed in the present study (Tables 1 and 3).

To the best of our knowledge, the impact of physiological UCB concentrations in vivo on oxidative damage to DNA assessed by SCGE, as well as the measurements of urinary 8oxodGuo were not investigated before in mammalian organisms. So far, only one very recent study reported lower 8oxodGuo levels in male Gilbert syndrome individuals and compared them to healthy controls, indicating reduced oxidative DNA damage (15). Their finding indicates a role for bilirubin in preventing oxidative damage to DNA; however, the results of the present investigation do not support such a conclusion, also when only men were considered (Table 1). Furthermore, Chang and colleagues (19) analyzed the relationship between polymorphisms of \textit{UGT1A1} and endogenous DNA damage (assessed by SCGE) as well as the repair capacity and radiation sensitivity. They found no association between DNA damage and \textit{UGT1A1}. This finding is in agreement with our results. A lower repair capacity and radiation sensitivity was seen in individuals with the \textit{UGT1A1*28} polymorphism. Khan and Poduval (31) reported induction of strand breaks in human lymphocytes after incubation with 50 \( \mu \)mol/L UCB and subsequent radiation increased the comet tail even more (31). Importantly, a recent Japanese (13) and an Austrian (14) in vitro study with human carcinoma cell lines indicate anti-genotoxic effects of bilirubin, by induction of typical comet tails. Moreover, bilirubin leads to cell-cycle arrest and subsequent apoptosis in various cancer cell lines (13, 14).

Furthermore, bilirubin inhibited the mutagenic effect of pro-oxidative tertiary-butyl hydroperoxide (t-BuOOH) in the Salmonella/microsome assay (12). Speit and colleagues showed that the induction by hyperbaric oxygen (HBO) treatment of heme oxygenase 1 (HO-1), which is the rate-limiting enzyme in bilirubin production, prevents oxidative damage to DNA. These findings indicate that the increase of the UCB concentrations may account for the adaptive response (32). In this context, it is notable that the “hormesis theory” might be relevant in explaining chronic effects of mild hyperbilirubinemia. It is already proven for some antioxidants that they assist in the prevention of long-term diseases. These antioxidants act as “low dose stressors” and may prepare cells to resist more severe stress (33). This theory might explain the protective effects of bilirubin in epidemiologic investigations (7, 11), however, not in the present cross-sectional study.

Results of lifestyle interventions on biomarkers of oxidative DNA and RNA damage are inconsistent (34, 35). Scientific reports on RNA oxidation are limited compared to that on oxidative DNA damage. While, Giovanelli and colleagues did not find associations between biomarkers assessed by SCGE assay and age, sex, and BMI (35), an effect of age on DNA damage (8oxodGuo; ref. 34) and RNA damage (8oxoGuo; ref. 36) has already been published. The present results (Table 2) do not show an association with age and 8oxodGuo but by trend for 8oxoGuo (\( P = 0.067 \)). In regard to sex, it was also shown that males have greater DNA damage when compared with females (37), due to higher metabolic rate in men (38). Men also suffer more often from cancer than women (39). The present study revealed a lower oxidative DNA damage (8oxoGuo, \( P < 0.05 \)) but also a trend for higher strand breaks (\( P = 0.060 \)) in men. Moreover, greater damage to DNA (assessed by SCGE) with increasing BMI in females was reported (40). Importantly, we found that the UCB levels were lower (\( P < 0.05 \)) and strand breaks were higher (\( P = 0.064 \)) in the higher BMI group (\( \geq 25 \text{ kg/m}^2 \)), leading to the assumption that UCB might be associated with the body weight and also with damage to DNA, which, however, has to be further explored. This was also indicated by a negative association between UCB and BMI. The impact of lipid metabolism and a generally lower lipid profile in Gilbert syndrome subjects might also be related to the lower BMI, what was recently shown by our group (16, 41). Another observation showed that weight loss was associated with increasing bilirubin levels and was more likely in men (42). Obesity leads to increased oxidative stress (43), as adipose tissue is one major source of elevated plasma oxidants (44) and might also be linked to higher insulin resistance in this group (45).

Surprisingly, lower 8oxodGuo concentrations were detected in the higher BMI group (Table 2), which was also confirmed by a negative correlation between both variables. The same association was seen in a previous study (37), and it was hypothesized that the higher metabolic rate in lean subjects is responsible for this finding, suggesting that oxidative damage to DNA mainly occurs in the lean body fraction (46). Furthermore, the concentrations of 8oxodGuo [1.86 nmol/mmol creatinine (0.52)] are comparable with results from another Austrian study [8oxodGuo 2.00 nmol/mmol creatinine (0.75); ref. 47].

Sufficient folic acid and vitamin B12 concentrations are crucial for DNA biosynthesis and methylation (48) and are important for prevention of DNA damage (49). In the present study, vitamin B12, but not folic acid, was negatively correlated to DNA/RNA damage (strand breaks, FPG-sensitive sites, and 8oxoGuo).

The relatively young and healthy population studied here might have limited the detection of significant differences between normo- and hyperbilirubinemic individuals. However, elevated oxidative stress is also detectable in young populations (50), and protection from oxidative stress was shown in a relatively young male Gilbert syndrome group (15), but a DNA protective effect might be more readily detected in older populations where the levels of DNA damage and oxidative stress might be greater.

Taken together, this study did not reveal a protective effect of chronically elevated UCB on biomarkers of DNA or RNA.
oxidation. Hyperbilirubinemic subjects had a lower BMI which might indicate an indirect effect of UCB in health promotion. Other plasma antioxidants were not associated with oxidative DNA/RNA damage, except vitamin B12. However, further investigations on bilirubin’s anti-genotoxic effects are of high importance to reveal mechanistic explanations for the existing association between bilirubin and oxidative stress–related diseases including cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Prof. O. Wagner and A/Prof. M. Wolzt from the General Hospital Vienna for their valuable support, R. Quin, PhD, from the Department of Nutritional Sciences in Vienna for conducting the γ-irradiation, D. Toferer for measuring homocysteine levels, and M. Gierer for measuring vitamin C, β-carotene, and glutathione. The authors also thank T. Henriksen, PhD, from Laboratory of Clinical Pharmacology Copenhagen for conducting the 8oxodGuo and 8oxoGuo analysis and the clinic staff of the Department of Clinical Pharmacology for subjects’ care and performance of blood samplings.

Grant Support
This work was supported by the Austrian Science Fund (grant number P21162-B11; recipient K.-H. Wagner and A.C. Bulmer) and by the Vienna Science and Technology Fund (grant number I507-031; recipient D. Doberer).

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Received April 7, 2013; revised July 10, 2013; accepted August 5, 2013; published OnlineFirst August 27, 2013.

References
Anti-Genotoxic Potential of Bilirubin In Vivo: Damage to DNA in Hyperbilirubinemic Human and Animal Models

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