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DISSERTATION

Titel der Dissertation

„*Anti-oxidative* and *anti-genotoxic* potential of unconjugated Bilirubin“

verfasst von

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I. Abstract

Unconjugated Bilirubin (UCB), the product of heme catabolism, is known as a potent antioxidant. A mild elevation of circulating plasma UCB, also known as Gilbert`s syndrome (GS) is associated with a reduced risk of non communicable diseases (NCDs) such as cardiovascular diseases and cancer.

This study aimed to find explanations for the reduced disease risk observed in GS by exploring whether serum bilirubin concentrations are linked to changes in DNA damage, to altered lipid metabolism and inflammation status in human and animal models.

Therefore, a variety of biomarkers were investigated in a cross sectional case control study comparing healthy subjects with Gilbert`s syndrome to age- and gender matched controls (n= 38 per group, total n= 76). Changes in DNA oxidation were evaluated using the cytokinesis-block micronucleus assay (CBMN), buccal micronucleus cytome assay (BMcyt) and the single cell gel electrophoresis (SCGE) assay. In addition, urinary 8-oxo-2`-deoxyguanosine (8oxodGuo) for estimation of DNA oxidation and urinary 8-oxo-guanosine (8oxoGuo) that reflects RNA oxidation were measured. Moreover, the lipid profile (lipoproteins, triglycerides (TG), cholesterols, including subfractions of LDL) and biomarkers of inflammation (interleukin 6 (IL-6), IL-1β, tumor necrosis factor α (TNF-α), serum amyloid A (SAA), C-reactive protein (CRP)) as well as heme metabolites (UCB, carboxy hemoglobin (CO-Hb) and iron) were analysed.

In addition, a hyperbilirubinemic rodent model (Gunn rats vs. Wistar rats, n= 20 per group, total n= 40) was selected to support the data observed in the human study. Within this trial, changes in DNA damage were determined in different tissues by using the SCGE assay, together with assessing the lipid profile.

The GS group and the Gunn rats had significantly higher UCB concentrations and a lower body mass index (BMI; human data) or lower body mass (female Gunn rats only) compared to their respective controls ($p<0.05$). With reference to DNA damage no significant difference in GS subjects versus controls was observed. Surprisingly, significantly higher DNA strand breaks (measured in white blood cells, PBMCs) were identified in Gunn rats when compared to controls.

Relative to their controls GS subjects had a significantly improved lipid profile (reduced total cholesterol, LDL-cholesterol, TG, LDL subfractions (LDL-1+LDL-2), lipoprotein (Apo-B), Apo-B/Apo-A1 ratio ($p<0.05$). Moreover, lower IL-6 and SAA concentrations and higher IL-1β concentrations were found when compared to controls ($p<0.05$; $p=0.09$; $p<0.01$). These data were confirmed by lipid analyses in the rodent model, showing that Gunn rat serum contained lower total cholesterol and TG concentrations as compared to that of normobilirubinemic rats ($p<0.001$). Furthermore, individuals with GS had significantly higher levels of CO-Hb and iron ($p<0.01$; $p<0.001$). When lifestyle effects (age and BMI) were considered statistically, lower formation of buccal anomalies were found in the GS subgroup of ≥30 years compared to the GS subgroup of <30 years of age ($p<0.05$). GS subjects of ≥30 years of age had reduced lipid variables (total cholesterol and LDL-C, TG and LDL-C subfractions, Apo-B/Apo-A1 ratio, Apo-B) when compared to older controls ($p<0.05$). The group with a BMI ≥25 kg/m^2 had lower 8oxodG concentrations and tended to have higher DNA strand breaks. Simultaneously, these subjects had lower UCB levels than normal weight subjects ($p<0.05$; $p=0.064$; $p<0.05$).

These findings suggest DNA protection in older GS individuals` epithelia, however, a disease preventing effect which has been reported previously in a series of epidemiological studies, does not seem to be based on an immediate UCB-derived DNA protection. Consequently, the effects observed in PBMCs utilising the SCGE assay were not as pronounced. The link to a better health status (e.g. reduced risk of NCDs) observed in GS individuals, is probably rather predicted on the lower BMI, altered lipid profile and reduced inflammation status that were noticed in hyperbilirubinemic subjects, particularly in the older individuals. Therefore, it is likely that UCB, in addition to its commonly known antioxidant behavior, also exceeds its beneficial health effects via alternate mechanisms which are yet to be explored.

In this respect a newly hypothesised relationship between UCB, heme, iron and CO-Hb possibly indicates a positive feedback loop that includes UCB as a direct inducer of HO-1.

The results of the present study that are mainly based on observations, should be pursued in future, to follow-up on this and other hypotheses.

II. Zusammenfassung

Unkonjugiertes Bilirubin (UCB), das Abbauprodukt des Hämoglobinstoffwechsels, ist ein wirkungsvolles Antioxidans. Moderat erhöhte Blutbilirubinkonzentrationen (wie bei Gilbert`s Syndrom (GS)), sind assoziiert mit einem verminderten Risiko für chronische, nicht übertragbare Erkrankungen (NCDs) wie kardiovaskuläre Komplikationen und Krebs.

Die vorliegende Studie untersuchte, ob zirkulierendes Bilirubin mit Veränderungen von DNA Schäden, sowie mit einem veränderten Lipidstoffwechsel und Entzündungsstatus in Verbindung gebracht werden kann und ob daraus ein Erklährungsansatz für das verminderte Krankheitsbild abgeleitet werden kann. Dazu wurden eine Humanstudie und ein Tiermodell geplant und durchgeführt.

In einer Fall-Kontrollstudie im Querschnittdesign wurden eine Vielzahl an aussagekräftigen Biomarkern in Proben von Studienteilnehmern mit GS gemessen und mit den Ergebnissen entsprechender Kontrollpersonen verglichen (n= 38 pro Gruppe, gesamt n= 76; Alter und Geschlecht angepasst). Veränderungen der DNA Schädigung/Oxidation wurden mit dem Mikronukleus Test in peripheren Blutlymphozyten (PBLs) und Mundschleimhautzellen bestimmt. Weiters wurde die Einzelzellgel Eletrophorese (SCGE) angewandt und weitere Biomarker im Urin (8-oxo-2`-Deoxyguanosin (8oxodGuo, DNA Oxidation) und 8-oxo-Guanosin (8oxoGuo, RNA Oxidation) wurden hinzugezogen. Das Lipidprofil (Lipoproteine, Triglyzeride, Cholesterine, inklusive Subfraktionen des LDL-Cholesterins) und der Entzündungsstatus (Interleukin 6 und 1β (IL), Tumor Nekrose Faktor α (TNF-α), Serum amyloid A (SAA), C-reaktives Protein (CRP)) wurden analysiert sowie weitere Metaboliten, die zum Häm Stoffwechsel zählen (UCB, Carboxy Hämoglobin (CO-Hb) und Eisen).

Um die Daten der Humanstudie zu unterstützen wurde ein Tiermodell mit Gunn Ratten (erhöhtes Blutbilirubin) verwendet und mit Wistar Ratten (Blutbilirubin im Normalbereich, n= 20 pro Gruppe, gesamt n= 40) verglichen. Dazu wurde der SCGE Test durchgeführt und das Lipidprofil im Rattenserum bestimmt.

Die GS Gruppe und die Gunn Ratten hatten, verglichen mit den Kontrollgruppen, signifikant höhere UCB Konzentrationen und einen geringeren Körpermasseindex (BMI) bzw. eine geringere Körpermasse bei den Ratten, allerdings nur bei den weiblichen ($p<0.05$). Darüber hinaus wurden keine weiteren signifikanten Unterschiede bezüglich der DNA Schädigung von Studienteilnehmern mit GS verglichen mit der Kontrollgruppe festgestellt. Überraschenderweise hatten die Gunn Ratten im Vergleich zu den Kontrollratten signifikant höhere Strangbrüche in einkernigen Blutzellen des peripheren Blutes (PBMCs, $p<0.05$).

Nach Analyse des Lipidprofils stellte sich heraus, dass Individuen mit GS reduzierte Konzentrationen an Gesamtcholesterin, Triglyzeriden, LDL Subfraktionen (LDL-1 und LDL-2), Lipoproteinen (Apo-B) und ein vermindertes Verhältnis aus Apo-B zu Apo-A1 aufwiesen ($p<0.05$). Außerdem wurden reduzierte IL-6 und SAA Konzentrationen sowie erhöhte IL-1β Level im Vergleich zu der Kontrollgruppe gefunden ($p<0.05$; $p=0.09$; $p<0.01$). Diese Daten wurden durch die Lipidanalyse der Nager unterstützt, da die Gunn Ratten ebenfalls reduzierte Gesamtcholesterin- und Triglyzerid Werte aufwiesen verglichen mit den Wistar Ratten ($p<0.001$). Weiters wurde festgestellt, dass Personen mit GS höhere CO-Hb- und Eisenkonzentrationen aufwiesen ($p<0.01$; $p<0.001$). Auch Lebensstileffekte (Alter und BMI) wurden untersucht und es stellte sich heraus, dass die GS Gruppe mit einem Alter von ≥30 Jahren, verglichen mit der jüngeren GS Gruppe, niedrigere chromosomale Schädigungen in den Epithelzellen aufwiesen ($p<0.05$). Bei der gleichen Gruppe (GS ≥30 Jahren) wurden auch verringerte Level an Gesamt- und LDL-Cholesterin, Triglyzeriden, LDL-Subfraktionen, Apolipoprotein B1 und ein vermindertes Verhältnis aus Apo-B zu Apo-A1 festgestellt verglichen mit der älteren Kontrollgruppe ($p<0.05$). Niedrigere 8oxodGuo Konzentrationen ($p<0.05$) wurden in der Gruppe mit einem BMI ≥25 kg/m^2 gemessen, obwohl diese Studienteilnehmer niedrigere Bilirubinwerte hatten als normalgewichtige Teilnehmer ($p<0.05$).

Diese Resultate deuten eine DNA schützende Wirkung in epithelialem Gewebe von älteren Individuen mit GS an. Allerdings ist ein krankheitspräventiver Effekt, wie er in epidemiologischen Studien gezeigt wurde, wohl nicht auf eine DNA schützende Wirkung

von Bilirubin (gemessen in PBMCs) zurückzuführen. Das veränderte Lipidprofil und der reduzierte Entzündungsstatus von Personen mit erhöhten Bilirubinspiegeln, besonders von älteren Individuen, tragen möglicherweise, zusätzlich zu den bereits bekannten antioxidativen Effekten von Bilirubin zum Gesundheitsschutz bei. Die Beziehung zwischen UCB, Häm, Eisen und CO-Hb weist auf eine positive Rückkopplungs Schleife (feedback loop) der Hämoxygenase hin, die eventuell über Bilirubin vermittelt wird. Abschließend, ist zu vermuten, dass der reduzierte BMI in der GS Gruppe möglicherweise indirekt zu einem reduzierten Risiko an NCDs beiträgt.

Die vielversprechenden Ergebnisse der vorliegenden Arbeit, die hauptsächlich auf Beobachtungen beruhen, erfordern weiterführende Studien auch auf mechanistischer Ebene.

III. List of Publications

The present thesis is based on the following articles:

Original Article 1:

Wallner, M., Blassnigg, S. M., Marisch, K., Pappenheim, M. T., Mullner, E., Molzer, C., Nersesyan, A., Marculescu, R., Doberer, D., Knasmuller, S., Bulmer, A. C. and Wagner, K. H. **Effects of unconjugated bilirubin on chromosomal damage in individuals with Gilbert's syndrome measured with the micronucleus cytome assay**. Mutagenesis. 2012;27:731-5.

Original Article 2:

Wallner, M., Antl, N., Rittmannsberger, B., Schreidl, S., Najafi, K., Mullner, E., Molzer, C., Ferk, F., Knasmuller, S., Marculescu, R., Doberer, D., Poulsen, H. E., Vitek, L., Bulmer, A. C. and Wagner, K. H. **Anti-genotoxic potential of bilirubin in vivo: damage to DNA in hyperbilirubinemic human and animal models.** Cancer prevention research. 2013;6:1056-63.

Original Article 3:

Wallner, M., Marculescu, R., Doberer, D., Wolzt, M., Wagner, O., Vitek, L., Bulmer, A. C. and Wagner, K. H. **Protection from age related increase in lipid biomarkers and inflammation contributes to cardiovascular protection in Gilbert's syndrome.** Clin Sci (Lond). 2013; 125:257-64.

Original Article 4:

Wallner, M., Bulmer, A. C., Molzer, C., Mullner, E., Marculescu, R., Doberer, D., Wolzt, M., Wagner, O. F. and Wagner, K. H. **Heme catabolism: a novel modulator of inflammation in Gilbert's syndrome.** Eur J Clin Invest. 2013;43:912-9.

Reprints and accepted papers are part of this thesis, with kind permission of respective publishers.

IV. Contents

V. Figures

VI. Tables

VII. Abbreviations

8oxodG	8-oxo-7,8-dihydro-2´-deoxyguanosine
8oxodGuo	8-oxo-guanosine
ALP	Alkaline phosphatase
ALT	Alanine transferase
Apo A/B1	Apolipoprotein
Apo	Apoptosis
AST	Aspartate aminotransferase
BMcyt	Buccal micronucleus cytome assay
BMI	Body mass index
BN	Binucleated cell
BR	Bilirubin
BV	Biliverdin
CAD	Coronary artery disease

CBMN	Cytokinesis block micronucleus assay
CC	Condensed chromatin
cGMP	Guanosine 3´, 5´-cyclic monophosphate
CN	Crigler Najjar syndrome
CO	Carbonmonoxide
CO-Hb	Carbonmonoxide bound to hemoglobin
COPD	Chronic obstructive pulmonary disease
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmatic reticulum
FAD	Flavin adenine dinucleotid
FeII	Iron
FMN	Flavin mononucleotide
GS	Gilbert`s syndrome
GSH	Glutathione
GSSG	Oxidized glutathione
HBO	Hyperbaric oxygen
HDL C	High density lipoprotein cholesterol
HDL	High density lipoprotein
HO	Heme oxygenase
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease
IDL	Intermediate density lipoprotein
IHD	Ischemic heart disease
IL	Interleukin
KL	Karyolyses
KR	Karyorrhexis
LDH	Lactate dehydrogenase
LDL C	Low density lipoprotein cholesterol

LDL	Low density lipoprotein
LMA	Low melting agar
MHC	Major histocompatibility complex
MNi	Micronucleus
NADPH	Nicotinamide adenine dinucleotide phosphate
NBud	Nuclear bud
NCDs	Non communicable disease
Necr	Necrosis
NPB	Neoplasmatic bridge
OGG1	Oxoguanine glycosylase
P	Pycnotic cells
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononucleated cells
RNA	Ribonucleic acid
ROS/RNS	Reactive oxygen/nitrogen species
SCGE	Single cell gel electrophoresis
TC	Total cholesterol
TG	Triglycerols
TH1/TH2	T lymphocytes, helper cells
TNF	Tumor necrosis factor
UCB	Unconjugated bilirubin
UGT1A1	Uridine diphosphate glucuronosyl transferase
UGT1A1*28	Homozygous genotype, typical for GS
VLDL	Very low density lipoprotein
γ-GT	Gamma glutamyl transferases

1. Literature Review

1.1. Unconjugated bilirubin: chemistry, characteristics and metabolism

The bile pigment unconjugated bilirubin (UCB), is derived from heme (iron-protoporphyrin IX) catabolism in the human body. The porphyrin is characterized by a tetrapyrrolic structure, that, as a consequence of intramolecular H-bonds, leads to a poor solubility in water (Figure 1). Especially, the vinyl and methene groups in the structure of UCB contain double bonds that are easily oxidized and highly photosensitive, leading to limitations in its handling as a pure compound. Nevertheless, particularly this specific property leads to the important function of UCB as an antioxidant (2). Bilirubin is a lipophilic compound with hydrophilic carboxyl groups (3). These groups are unreachable for water due to intramolecular H-bondings to pyrrolic amino groups attached to BR (4). This conformation of bilirubin has a high affinity to nervous tissue (5).

In fact, only moderate elevated unconjugated bilirubin concentrations of up to about 102.6 µmol/l (6 mg/dl; as found in Gilbert`s syndrome) may act in a beneficial way, values above these levels are to be considered in a pathophysiological context (6).

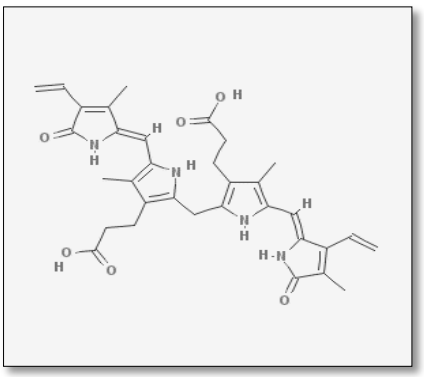

Figure 1: IX-α-Bilirubin

As mentioned above, UCB is a product of the heme catabolic pathway (Figure 2). 300 to 400 mg bilirubin are generated endogenously per day of which around 80 % result from the breakdown of erythrocytes (7). The cleavage of the α-methene bridge (8) by

microsomal heme oxygenases (HO) 1 or 2 leads to the formation of biliverdin (BV) and release of iron (Fe II) and carbon monoxide (CO). NADPH-cytochrome P-450 reductase together with FAD and FMN is required for this reaction (9). Then, at C-10 location the central methene bridge of biliverdin is reduced to UCB by NADPH-biliverdin reductase (10). For the transport to the liver, lipophilic UCB is bound to albumin and in the cytosol of hepatocytes bilirubin binds to ligandin and is moved to the endoplasmatic reticulum (11). There, the conjugation is catalyzed by the enzyme uridine diphosphate glucuronosyl transferase (UGT1A1) to generate conjugated bilirubin. UGTs (UGT1 and UGT2) are furthermore important for the conjugation of bile acids, xenobiotics and steroids (5). It is hypothesized that the glucuronidation is located on the hydrogen-bonded conformation of bilirubin (12). The binding of one or two molecules of glucuronic acid or sugar molecules (xylose and glucose) leads to the formation of watersoluble bilirubin mono- or diglucuronides (11, 13). The subsequent excretion into the bile functions via an ATP-dependent anion transporter (cMoat) (5, 14). The excretion of glucuronides was suggested to be rate-limiting in bilirubin transfer (5).

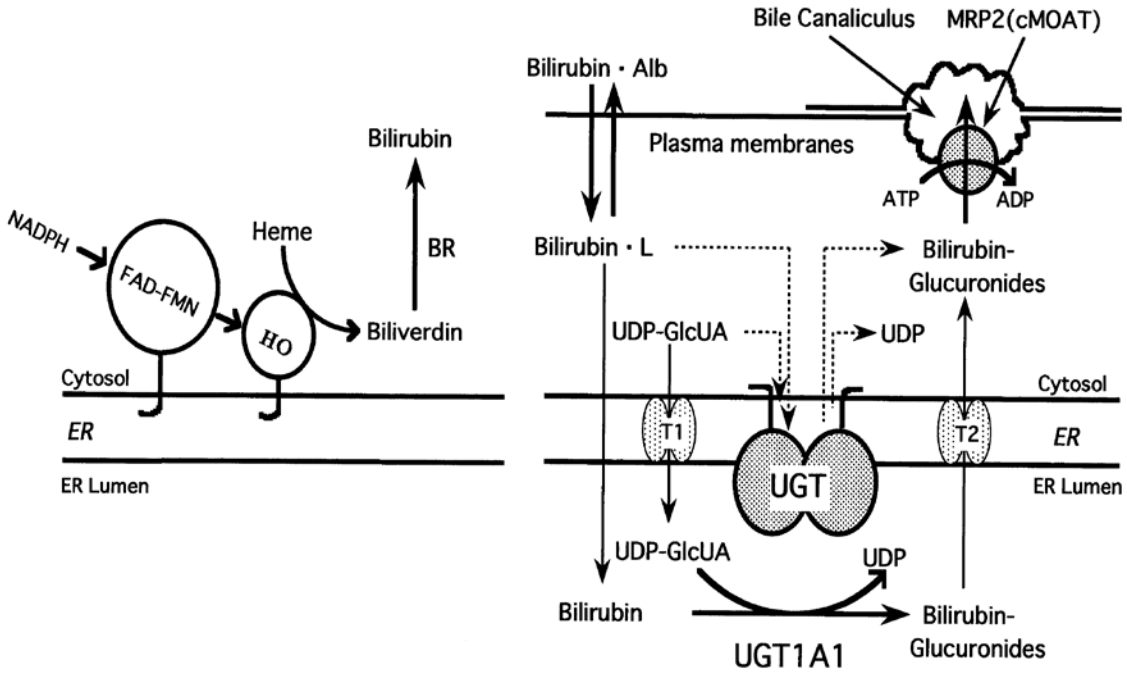

Figure 2: Metabolic pathway from heme to bilirubin-glucuronides (5)

Abbreviations: FAD,FMN, NADPH-cytochrome P-450 reductase, HO, heme oxygenase, BR, biliverdin reductase; UGT, UDP-glucuronosyltransferase; Bilirubin-Alb, bilirubin albumin complex, MRP, multidrug resistance protein 2; UDP-GlcUA, UDP-glucuronic acid, T1 and 2 are proposed transporters in ER membrane, dashed lines show alternate pathway for glucuronidation in ER

1.2. Moderate hyperbilirubenemia in humans: Gilbert`s syndrome

As indicated above, Gilbert`s syndrome is a benign condition resulting in moderately elevated unconjugated bilirubin serum concentration in the absence of underlying liver diseases and hemolysis. The condition is partly characterized by an abnormality in the UGT1 gene complex. The homozygous polymorphism of the first exon (1A1) is typical for subjects with GS. The protein encoded by UGT1A1 is generally responsible for bilirubin glucuronidation. Especially, a modification in the TATAA element with two additional TA nucleotides ($A(TA)_7TAA$) in the 5` promoter region of the UGT1A1 gene leads to a reduced expression of UGT1 and correlates with significantly higher UCB levels as compared to the heterozygous genotype ($A(TA)_6TAA$) (15). The hepatic activity of UGT1A1 is reduced to approximately 30 % of the normal enzyme function (16). Previous reports also indicate a certain influence of an additional genetic variability apart from that affecting glucuronidation, as the clearance for tolbutamide (not metabolized by glucuronidation) was found to be abnormal in GS but not in hyperbilirubinemic subjects with diagnosed hemolysis (17). Glucuronidation is also involved in the excretion of substances other than bilirubin that might affect the metabolism of therapeutic drugs and substrates in GS. If UGT1A1 is the isoform responsible for glucuronidation such as for irinotecan (antitumor agent) (18) and atazanavir (19), impaired metabolism of these drugs might result in toxicity. Furthermore, 2-hydroxy-estrone, estradiol and ethylestradiol (20) are glucuronidated via this pathway as well as other drugs like paracetamol and oxazepam (21). Administration of the microsomal enzyme inducers phenobarbital (22) or rifampicin (23) to subjects with hyperbilirubinemia leads to an increased enzyme activity to elevate the bilirubin excretion in case of severe forms (Crigler najjar type II) and also, but not necessarily, in subjects with GS.

Importantly, the “caloric restriction intake” test is a suitable diagnostic procedure for GS as its genetic polymorphism is correlated with UCB levels. The intake of not more than 400 kcal within 24 h almost doubled UCB levels in individuals with GS but not in patients with liver disease or hemolysis and healthy subjects (24). Gilbert`s syndrome is defined by circulating UCB levels greater than 17.1 µmol/l (1 mg/dl) and up to

102.6 µmol/l (6 mg/dl) in the absence of underlying liver disease or hemolysis. Thus, the waiver of genetic testing for GS diagnosis is common in the literature (25-28).
Interest in GS and its potential to prevent diseases (see below, chapter 1.5) has risen during the past decades, which is probably also due to the syndrome`s high prevalence. The polymorphism affects approximately 3-13 % of the general population (~12.4 % men, ~4.8 % women) (15, 29, 30). Men have higher UCB levels compared to women which is caused by the androgen steroid inhibition of bilirubin glucuronidation (30).

1.3. Animal Model: hyperbilirubinemic Gunn rats

The Gunn rat is a spontaneous mutant strain bred out of Wistar rats and is characterised by hyperbilirubinemia. The Gunn rat was first described by Gunn in 1938 as yellowish with abnormally high levels of bile pigments. Some of the pups already had a yellow complexion at birth, some of them only developed the colour 12 hours after delivery. Further observations included the hereditary autosomal recessive condition, that was displayed as 100 % jaundiced rats (homozygous, jj) from bred out of jaundiced males and females, whereas nearly 50 % of the litter from homozygous and affected animals were icteric (31). In experiments it is of advantage to use unjaundiced littermates (heterozygous, Jj) as matched controls. Deficiency in UGT activity leads to severe unconjugated hyperbilirubinemia and is mainly comparable with the human Crigler-Najjar syndrome (CN) Type 1 (5), in which the enzyme`s activity is lacking completely (32) and blood UCB concentrations range between 350 and 800 µmol/l (33). The high levels of UCB lead to neurotoxic effects including cell loss and gliosis in the cerebellum, hippocampus and basal ganglia (34).
The Gunn rat was used as animal model in the present thesis, as no alternative mild bilirubinemic rat model exists so far to study hyperbilirubinemia as it occurs in GS. We observed that the UCB levels of the Gunn rats in our experiment varied between 71 µmol/l and 169 µmol/l. Although, some homozygous animals had UCB concentrations of above the GS range (up to 103 µmol/l; see chapter 1.2.) 12 out of 20 animals were within this range.

1.4. Oxidative Stress and its targets

In a healthy body, the antioxidant defense is used as a detoxification mechanism against reactive oxygen and nitrogen species (ROS, RNS). An imbalance between pro- and antioxidants to the disadvantage of antioxidants is known as oxidative stress (35). Oxidants, such as reactive oxygen and nitrogen species (ROS, RNS) are produced constantly in living organisms and further originate from external sources (36-38).

Particularly, hydroxyl (OH•), superoxide (O_2•-), nitric oxide (NO•) and peroxyl (RO_2•) radicals are active ROS/RNS. Usually, also nonradicals as peroxynitrite (ONOO-), hyperchlorus acid (HOCl), hydrogen peroxiode (H_2O_2) and ozone (O_3) are included in this definition, since they are able to induce free-radical reactions (38). The superoxide radical is built mainly during cell respiration in the mitochondria, within the P450 cytochrome system (39), and also during body`s defense against microorganisms in phagocytes (40, 41). Phagocytes further produce H_2O_2 and HOCl (38). (Figure 3)

Oxidative stress is thought to be involved in the onset of human diseases including cardiovascular diseases, cancer and inflammatory disorders and also in the ageing processes (38).

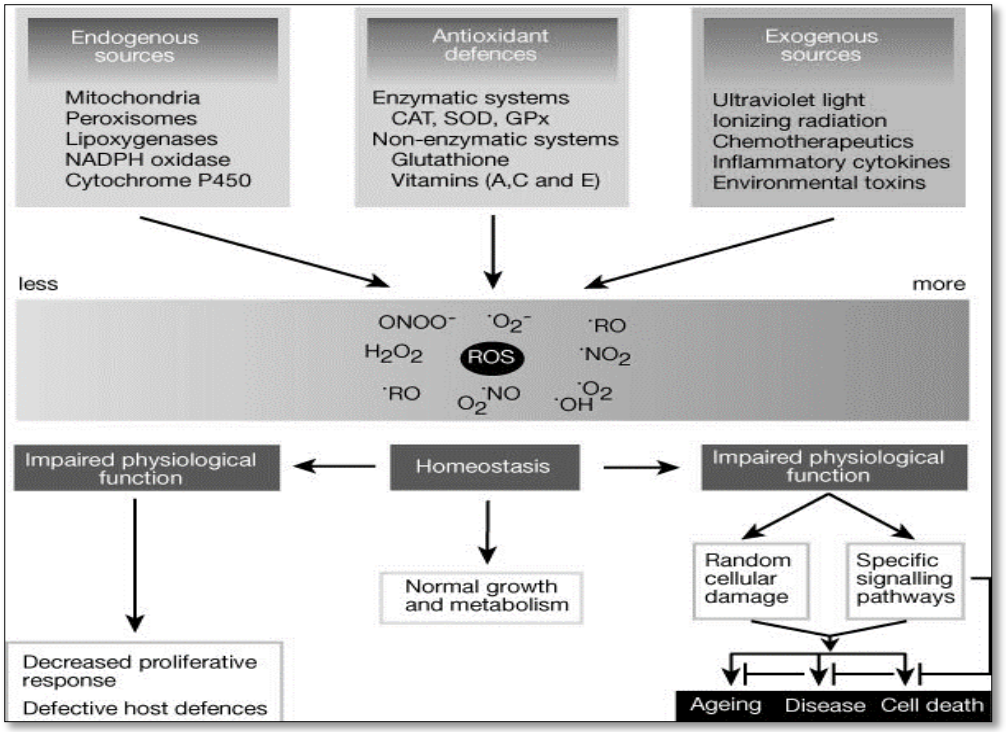

Figure 3: Oxidative stress; sources and consequences (35)

1.4.1. Oxidative damage to DNA and chromosomes

Reactive oxygen species interact with the DNA by oxidizing its bases (purines and pyrimidines) and abasic sites which further lead to single (SSB) and double (DSB) strand breaks and alkali labile sites (42, 43). At this stage repair of modified bases is likely as the cell provides several repair mechanisms, before replication is reached (44). Failed repair or unrepaired lesions of DNA might lead to damages in chromosomes undergoing replication. The question is, whether oxidative DNA damage is linked to cancer?

To date several articles have been published on this topic (43, 45). Only a part of them showed a link between oxidatively modified DNA products like 8oxodG and cancers such as colorectal cancer (46), leukemia (47) and lung-cancer (48), but still, large-scale epidemiological investigations are missing (49). However, the importance of measuring DNA damage is emphasised as studies connect oxidatively changed DNA to cardiovascular disease, diabetes and diseases of the nervous system as summarized recently (41). Furthermore, the association between chromosomal damage and cancer has already been proven (50) (Figure 4).

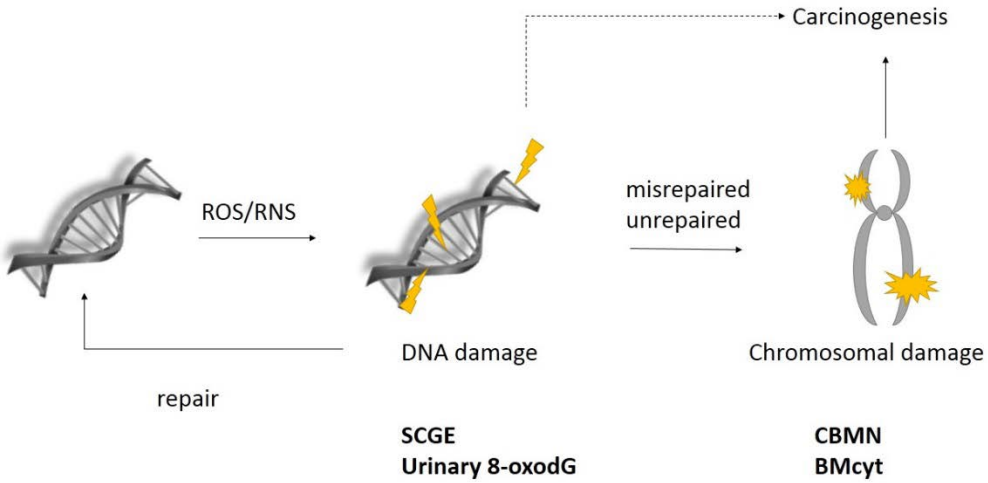

Figure 4: DNA oxidation and methods of detection

adapted after Sasaki (http://www.cometassayindia.org/introduction.htm)

1.4.1.1. From damage to DNA...

Common biomarkers for measuring oxidative DNA damage include the direct assessment of oxidized bases, in particular 8-oxo-7,8-dihydro-2´-deoxyguanosine (8-oxodG) and the markers of the single cell gel electrophoresis (SCGE) in which single- and double strand breaks as well as oxidized purines and pyrimidines can be determined.

Mechanisms of the formation of oxidatively modified DNA

High interest gains the C-8 modification of guanine to estimate oxidative damage to DNA (41, 42, 51). Upon the attack of a hydroxyl radical hydrogen is cleaved and results in the oxidation product 8oxodG (52). Due to its low ionisation potential guanosine is highly instable and therefore the most investigated product of DNA oxidation (53). Further known oxidation products of DNA bases are 8-oxoadenine, 2-hydroxy adenine, 5-hydroxymethyluracil, 5-hydroxycytosine, cytosine and thymine glycol and many more modification products have already been identified (42, 54). The formation of a particular modification is dependent on the ROS involved. Singlet oxygen mostly leads to the formation of 8-oxodG (55) whereas, superoxide anions are not reactive enough for this modification (56). The most powerful of all radicals in terms of genetic modification processes is the hydroxyl radical (42), which leads to DNA damage due to its addition to double bonds of DNA bases or the abstraction of hydrogen or hydrocarbons (57).

In the light of these reports, the constant repair of damaged DNA is important as different enzymes anneal strand breaks, excise modified bases and insert intact nucleotides (58). Especially, Endonuclease III is able to repair oxidized pyrimidines since it has glycosylase and endonuclease activitiy (59). The formamidopyrimidine-DNA glycosylase (FPG) and oxoguanine glycosylase (OGG1) are important enzymes to eliminate 8oxodG and other oxidized purines (60, 61). The oxidized repair products are mostly excreted via urine. An inadequate repair capacity results in elevated mutations which might stimulate carcinogenesis and thereby trigger the development of cancer and other diseases (41, 62). A prospective study was conducted on 54 000 subjects and it was shown that urinary excretion of 8oxodG was associated with increased lung-

cancer risk among never-smokers (63). In contrary a study on breast cancer did not show different 8-oxodG levels in cases versus controls (48).

1.4.1.2. ...to mutated chromosomes

Anomalies in chromosomes are commonly measured in peripheral blood lymphocytes (PBLs) by the cytokinesis-block micronucleus cytome assay (CBMN (64)) and in buccal cells by the buccal micronucleus cytome assay (BM-Cyt (65)). In addition to micronuclei (MNi) which reflect structural and chromosomal aberrations (66) other DNA instabilities such as nuclear buds (NBuds), nucleoplasmic bridges (NPBs) and binucleated cells (BN) can be scored. In order to collect information about cytotoxicity the rate of apoptosis (apo), necrosis (necr), karyolysis (KL), karyorrhexis (KR), condensed chromatin (CC) and pycnotic (P) cells can be estimated. Especially, MNi, NPBs and NBuds are investigated most frequently and strongly discussed regarding cancer prediction (67).

Mechanisms for chromosomal changes

Multiple mechanisms lead to the formation of MNi (Figure 5). Most MNi originate from acentric chromosomes or chromatid fragments and also lost chromosomes during anaphase might lead to MNi (68). The direct development of acentric chromosomes from double strand breaks is rare and only likely when the repair capacity is too low (69). Instead, the misrepair of double strand breaks rather leads to the formation of symmetrical and asymmetrical chromosomes and fragments and further to MNi (64, 70). Acentric chromosomes may further arise from the coincidental excision repair of damaged bases (71). The hypomethylation of cytosine is thought to be a mechanism of malsegregated chromosomes (72). Further mechanism include defects in mitotic spindle assembly, mitosis check point and abnormal centrosome amplification and also dicentric chromosomes might be involved in chromosome loss (73, 74). Also, MNi might appear after breakage of NPBs during telophase (75).

The development of NPB normally occurs during anaphase when centromers of dicentric chromosomes are pulled to the opposite poles and fail to break. Then, the membrane is simply formed around and the connection between chromosomes is

visible as a bridge (64). A NPB might also originate from misrepaired DNA breaks and is then often associated with MNi (76) (Figure 5).

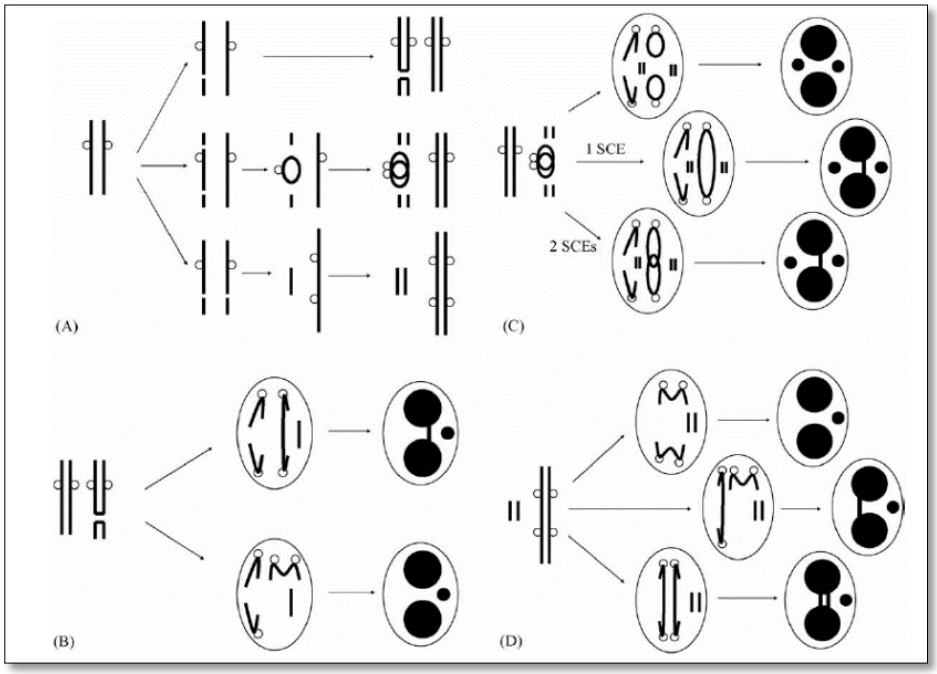

Figure 5: Development of MNi (77)

NBuds arise from amplified DNA that is eliminated from the nucleus via budding during the S phase of the cell cycle (78). Furthermore, breakage of a NPB and subsequent shrinkage of the leftovers lead to NBud formation (79).

It has been shown earlier that micronuclei (MNi) in lymphocytes are a robust marker for cancer rate and a relationship between increased MNi frequency and the incidence of lung (66), urogenital and gastro-intestinal cancers (50) has been documented. The association between MNi in lymphocytes and cancer was followed up for 22 years. A total number of 6718 subjects from 10 countries were investigated and MNi frequency was found as a predictor for cancer risk (50). More recent findings suggest that MNi in buccal cells are also valid markers for human cancer risks (80).

1.4.2. Oxidative stress and the role of lipids in atherosclerosis

One of the main targets of ROS/RNS, besides DNA and proteins, are lipids (81). The unpaired electron of a free radical can react with a fatty acid or acyl chain, subsequently

starting a chain reaction. The abstraction of a hydrogen atom, which possesses only a single electron, from a molecule, initiates the chain reaction of lipidperoxidation (Figure 6) and can result in the production of multiple lipid hydroperoxides.

$$L\text{-}H + OH\bullet \rightarrow H_2O_2 + L\bullet$$

Figure 6: Lipidperoxidation (1)

The chain reaction can be inhibited by exogenous and endogenous antioxidants providing a hydrogen atom (82) like vitamin E (36) or UCB (83). The vitamin E radical thereby formed is regenerated by vitamin C. All latter antioxidants are of high importance for the prevention of cardio vascular diseases (CVD (36)).

Lipid peroxidation contributes to atherosclerosis as an important predictor of which is oxidised LDL that is formed in human blood vessels (84). Lipoproteins are transport systems for endogenous lipids composed of very low density lipoproteins (VLDL) containing apolipoprotein B, low density lipoproteins (LDL) which carries mainly cholesterol and high density lipoproteins (HDL). The density of lipoproteins is crucial for atherosclerotic processes. Particularly, the abundance of VLDL, intermediate density lipoproteins (IDL) and small dense LDL in the lipid profile is linked to an increased atherosclerosis risk, what is a major health care problem in western countries (85, 86). Lesions in arterial walls arise as fatty streaks and are followed by accumulation of immune derived macrophages which are responsible for the uptake of cholesterol. It is indicated that oxidative modification of LDL particles (ox LDL) are responsible for initial fatty streak formation (87). The migration of monocytes, differentiating to macrophages might be stimulated by adhesion molecules such as VCAM-1 (88), E- and P-selectin (89).

However, there is evidence that oxLDL itself might be responsible for monocyte recruitment (84). Extensive amounts of cholesterol (transported by lipoproteins) are

found in macrophages leading to foam cells found in atherosclerotic lesions (90) (Figure 7).

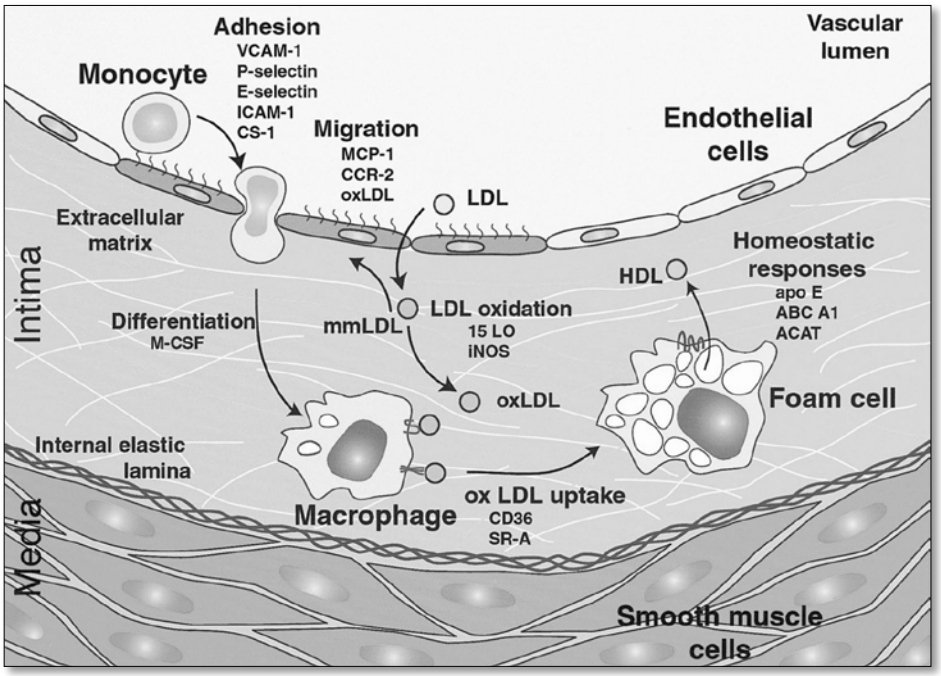

Figure 7: Initiation of atherosclerotic lesions (86)

Further immune response is activated by the expression of MHC class II molecules of foam cells leading to TH1/TH2 differentiation and the release of inflammatory cytokines like TNF-α and IL-6 (91). A rupture of atherosclerotic plaque in advanced lesions can lead to cardiovascular complications such as myocardial infarction (92).

1.4.3. Oxidative stress and HO-1 induction during inflammatory processes

Increasing evidence exists that HO-1 might mediate the cellular and systemic defense against oxidative stress (93). Therefore, the expression of HO-1 may be seen as a marker of oxidative stress (94). It was reported that the induction of HO-1 in lipopolysaccharides (LPS) stimulated mice led to decreased inflammatory lung injury (95). HO-1 was also found in foam cells and atherosclerotic lesions of apo-E-deficient mice and might thereby contribute to protection from atherosclerosis (96). Heme oxygenase-1 (previously described in chapter 1.1) is up-regulated by directly or indirectly formed ROS/RNS which may arise from UV radiation (97), heme itself (98),

proinflammatory cytokines, bacterial endotoxins (99) and tumor promotors (100). One mechanism for HO-1 activation might include chemical depletion of intracellularly reduced glutathione (GSH) (101)

Carbon monoxide, the by-product of heme degradation and HO-1 activity, is also investigated regarding possible health protection and not only considered as toxic agent, as it is mainly known e.g. as air pollutant (93). CO bound to hemoglobin (CO-Hb) at or above levels of 20 % lead to symptoms that include dizziness, breathing impairment, headache and may result in death when CO concentrations exceed 50-80 % (102). However, it was assumed that CO, as a small gas molecule similar to NO, is involved in cardiovascular protection and has anti-inflammatory properties (93). Indeed, renal ischemia/reperfusion led to an upregulation of HO-1 and increased levels of the signalling molecule guanosine 3´, 5´-cyclic monophosphate (cGMP), which is an important regulator of the vessel tone (103) particularly in the heart of rats (104). After rats were treated with CO, cGMP increased in vascular smooth muscle cells and might contribute to cardiovascular signalling (103). The mortality of HO-1 deficient mice increased after lung ischemia reperfusion. After subsequent CO inhalation, improved survival of HO-1 deficient mice was shown compared to HO-1 deficient mice without CO inhalation (105). LPS treated mouse leukeamic monocytes were prevented from TNF-α production after administering exogenous CO (106). As described above, the production/administration of CO is often tightly connected to HO-1 activation with release of bile pigments, assuming a positive feedback loop of CO and HO-1. Hence, protection from vascular disorders may be a result of this mechanism (107). Recently, germ free mice were experimentally infected with Salmonella typhimurium and HO-1 was activated by administration of a protoporphyrin complex. This procedure led to reduced bacterial counts and prevention of colonic inflammation. Exogenous administration of CO or overexpression of HO-1 increased pathogen defense in macrophages (108). It was hypothesised by Otterbein et al., that CO might not directly contribute to the defense system of macrophages. After administration it might increase defense resources including the inflammasome (109), which is a multiprotein complex of the innate immune system releasing IL-1β after caspase-1 activation (110)

and might contribute to bacterial defense mechanism (111). Mitochondrially produced ROS increase during stress conditions such as pathogenic invasion (112) and might lead to inflammasome activation (113). It is supposed that compensatory mechanisms are reduced to ensure increased levels of ROS assisting in defense (114). This might be achieved via the secretion of IL-1β supporting the neighboring cells to better resist against pathogen attacks (110).

1.5. Bilirubin and Gilbert`s syndrome in oxidative stress related diseases

Research around bilirubin has been expanding in the past decades. Reports show that the high interest in hyperbilirubinemia is justifiable however, it is important that bilirubin concentrations in blood are only moderately elevated in terms of health prevention. The analysis of blood bilirubin is already implemented in the routine analysis in many hospitals and preclinical laboratories which facilitates subject recruitment for clinical trials substantially.

1.5.1. Bilirubin and cancer

Already in 2001 an epidemiological study of the Belgian population (5460 men, 4843 women) reported that the risk for cancer mortality was significantly inversely correlated with bilirubin levels in men. These results were similar for women but did not reach significance (115). A chemopreventive function of bilirubin was also postulated in 2004 by American researchers in a study cohort of almost 17000 subjects. They found increasing bilirubin levels with decreasing cancer prevalence, especially for colorectal cancer (116). Horsfall et al. presented epidemiological data of more than 500 000 subjects of the UK primary care research data base. Bilirubin levels were associated with a lower risk of all-cause mortality and respiratory diseases, such as lung-cancer and chronically obstructive pulmonary disease (COPD) (117). A recently published Czech report revealed that carriers of the UGT1A1*28 allele were protected against colorectal cancer (CRC), this effect was more pronounced in men. They also reported that patients with CRC had significantly lower serum bilirubin levels than controls (118). Besides

these impressive effects of bilirubin on malignancy in humans, the underlying mechanisms remain still unclear.

1.5.2. Bilirubin and cardiovascular diseases (CVD)

Many groups investigated the CVD risk or markers associated with heart diseases and atherosclerosis on circulating serum bilirubin levels and individuals with Gilbert`s syndrome.

In 1994 Schwertner et al. already showed an increased risk for coronary artery disease (CAD) in subjects with low serum bilirubin levels (119). The results of the Framingham Heart Study including 4276 subjects demonstrated that increasing bilirubin levels were associated with fewer cardio vascular events, this result was more pronounced in men. Moreover, a subset of 1780 participants from the Framingham heart study were followed up for 24 years and genotyped for UGT1A1*28. The homozygous genotype showing high serum bilirubin had a lower risk for CVD (120).

With regard to CVD, a case control study on subjects with Gilbert`s syndrome (n=50), ischemic heart disease (IHD, n= 38) and controls (n ~2500) was conducted. Individuals with GS had a 10 % lower prevalence for IHD compared to the general population. Furthermore, subjects with GS had a higher total antioxidant capacity and HDL levels than controls (121). Boon et al. linked reduced circulating LDL, oxLDL and increased reduced thiol concentrations and relation of reduced to oxidized glutathione in GS subjects to a reduced risk for atherosclerosis (28). Furthermore, the increased antioxidant status and an improved resistance to serum oxidation in individuals with GS may partially contribute to the lower prevalence of CVD (122). The CVD risk and all-cause mortality among dialysis patients was investigated in a cohort study with 661 participants. Subjects with the UGT1A1 polymorphism (7/7 genotype) had a significantly decreased risk for CVD and all-cause mortality and it was speculated that elevated bilirubin levels in this group contributed to these tremendous effects (123).

1.5.3. Bilirubin and other diseases

In Crohn`s disease (n= 542), an inflammatory bowel disease, the UGT1A1 genotype was significantly less often detected as compared to the control group (n= 930). The authors

concluded that the higher serum bilirubin levels in the homozygous genotype may have been responsible for the protection of subjects from Crohn`s disease (124).

A very recently reported Japanese study with more than 2000 type 2 diabetic subjects reported an association between bilirubin and diabetic nephropathy (125).

1.6. Bilirubin in *in-vitro* studies and animal trials on proposed health promoting effects

The main protective mechanism of bilirubin is the antioxidant property to reduce oxidative stress (122, 126, 127).

A model for the regeneration of oxidised bilirubin was shown by Baranano et al. (128) who assumed that after bilirubin has scavenged ROS, the originated BV is again reduced to bilirubin by NADPH/biliverdin reductase (BVRA), similar to the GSH/GSSG redoxcycle by glutathionreductase. HO regulates the total levels of UCB and BV (Figure 8). In the above cited study, specifically BV was formed after in-vitro incubation of bilirubin albumin with 2,2`-azobis(2-amidinopropane) hydrochloride (AAPH) and after reaction of bilirubin with peroxyl radicals. It was also shown that 10 nM of bilirubin (IXα) protected HeLa cells from high H_2O_2 (10000 fold) toxicity but when these cells where deficient in BVRA three fold higher ROS levels were measured (128).

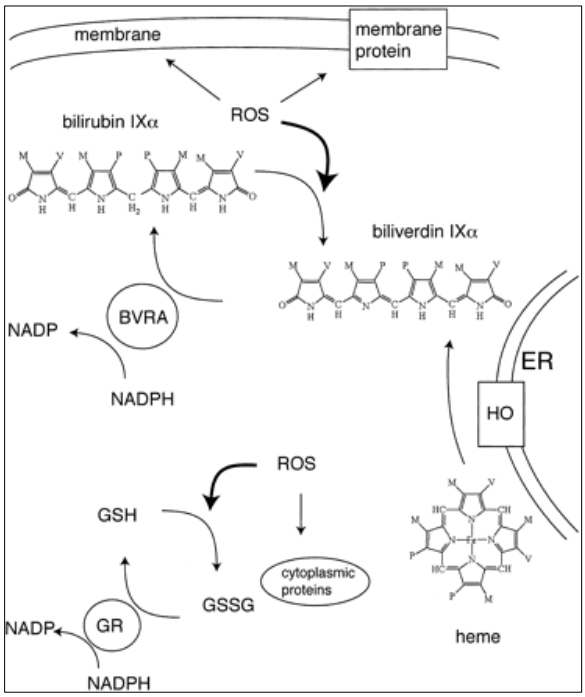

Figure 8: Biliverdin/Bilirubin redoxcycle - a model (128)

Already in 1987, Stocker et al. demonstrated the antioxidant effects of bilirubin. Linoleic acid (LA) was oxidised in an in-vitro system by a free radical chain reaction via 2,2`-azobis (2,4-dimethylvaleronitrile). A concentration dependent decrease of linoleic acid hydroperoxide was detected as doses of bilirubin/biliverdin increased. In a 2 % oxygen environment (corresponds to concentrations in tissues), bilirubin at a concentration of 10 µM inhibited the oxidation of linoleic acid by 35 %. When polyunsaturated fatty acids and phosphatidylcholine were used instead of LA, the inhibition of the reaction at 2 % oxygen increased to over 87 % with a better antioxidant performance of bilirubin compared to α-tocopherol (129).

Bilirubin reduced the mutagenic effect of pro-oxidative tertiary-butyl hydroperoxide (t-BuOOH) in the Salmonella/microsome assay (126). Furthermore, DNA damaging effects of different bile pigments on human cancer cell lines were tested with the SCGE assay. An increased content of % DNA in tail was measured, implicating an increased toxicity in human cancer cell lines (Caco2 and HepG2) and therefore anti-mutagenic properties of tetrapyrrols are assumed (130). In another experiment an increase of heme oxygenase 1 (HO-1) was induced by hyperbaric oxygen (HBO) treatment. HO-1 is the

rate limiting enzyme in bilirubin production and no further increase of oxidative DNA damage was induced by HBO treatment with increased HO-1 levels. These findings indicate that the increase in UCB concentrations may account for the adaptive response observed (131).

Another interesting effect, which was caused by UCB in *in-vitro* experiments, was the induction of tumor suppressor p53 (132) and hyperphosphorylation of the retinoblastoma tumor suppressor protein (Rb) (133), both effects which are causally related to the induction of apoptosis. In agreement with these results, it is also known that bilirubin induces apoptosis in cultured colon cancer cells (134).

Liver grafts were rinsed with a bilirubin solution which resulted in reduced biliary dysfunction and hepatic cell injury. The inhibitory function of bilirubin on lipid peroxidation in liver cells might have been responsible for these effects (135).

The administration of bilirubin to mice with LPS induced endotoxemia led to complete regeneration. Compared to control mice, after bilirubin injection the expression of immuno-regulatory genes encoding for Il-1β and TNF-α was reduced (136). The inflammatory effects of pyelonephritis in rats were reduced when a combination of bilirubin and antibiotics was administered to the animals for a short period (9 days) and a long period (6 weeks). Bilirubin alone was also able to prevent inflammation (at the long period only) and apoptosis (137).

The plethora of the results reported emphasizes the necessity of pursuing research in this field to obtain a more mechanistic insight into bilirubin`s properties in the body and to reveal how it might be able to reduce the risk for NCDs.

2. Hypotheses and Study Aims

This study aimed to compare results from subjects with elevated UCB levels on DNA damage and oxidative stress markers, inflammatory parameters and lipid profiles with respective controls. Furthermore, an animal trial was conducted to obtain information on DNA damage in different tissues and furthermore the lipid profile was analysed.

2.1. Human study (GS subjects vs. control subjects)

- To evaluate whether subjects with GS are better protected against DNA/chromosomal damage compared to age and gender matched controls. (Original Article 1 and Original Article 2)
- To evaluate whether subjects with GS are protected against cardiovascular risk factors (Original Article 3)
- To evaluate whether inflammatory markers are associated with HO-1 products (Original Article 4)

2.2. Animal trial (homozygous Gunn rats (hyperbilirubinemic) vs. heterozygous wistar rats (normobilirubinemic))

- To evaluate whether homozygous Gunn rats are protected against DNA damage compared to heterozygous Gunn rats (Original Article 2)
- To evaluate whether homozygous gun rats are protected against cardiovascular risk factors (Original Article 3)

3. Materials and Methods

3.1. Study design: Human study

A total number of 104 subjects were recruited from the general population. Seventy six subjects met the inclusion criteria as they possessed normal liver function, were aged between 20 and 80 years and were free from any underlying disease. Reticulocytes, γ-glutamyl transferase (γ-GT), alanine transferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), hemoglobin and hematocrit were measured at the screening examination. Subjects with liver, heart or kidney disease, hemeolysis, diabetes, cholelithiasis, organ transplants, history of CVD, cancer, smoking (>1 cig/day), alcohol consumption (>7 standard drinks/week), excessive physical activity (>10 h/week), any medication that might affect liver metabolism and vitamin supplementation (4 weeks prior the first blood sampling) were excluded. Allocation to the GS group was determined by a fasting serum UCB concentration of ≥17.1 μM measured by HPLC (see below). Subjects were age and gender (28 men, 10 women) matched in each group (total 76 subjects). This study was approved by the ethical committee of the Medical University of Vienna and the General Hospital of Vienna (# 274/2010) and performed according to the Declaration of Helsinki.

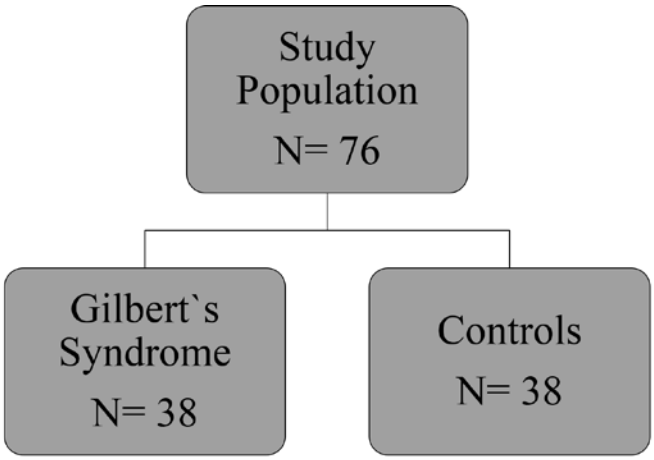

Figure 9: Study design of the human trial, age and gender matched controls

3.1.1. Sample preparations

A 24h fasting (400 kcal food restriction) blood sample was collected into lithium heparin and serum vaccutainers. Samples were stored on ice in the dark until further analysis. Blood collection tubes were centrifuged (15 min., 3000 rpm, 4°C) and serum or plasma was aliquotted, used fresh or stored at -80°C for further analysis. Erythrocytes from lithium heparin vaccutainers were washed three times with isotonic phosphate buffer, aliquotted and stored at -80°C.

3.2. Study design: Animal trial

Gunn rats (hyperbilirubinemic, homozygous for UGT1A1, jj) and respective controls (normobilirubinemic gunn rats, heterozygous for UGT 1A1 jJ) were obtained from Charles University in Prague (Prague, Czech Republic) and acclimatized in the breeding facility of the Medical University of Vienna (Himberg, Austria) 1 week prior to experiments. The animals were housed under standard conditions (24 ± 1 °C, humidity 50 ± 20 %, 12 h light/dark cycle) and fed with a standard diet (ssniff R/M-H Extrudat, ssniff Spezialdiäten GmbH, Germany). All experiments were carried out using 7-8 week old rodents (18 males, 22 females, total number of 40 rodents). The study was approved by the committee of animal experiments by 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 rodents) or a treatment group (11 jj and 11 jJ rodents). The treatment group was γ-irradiated (^{60}CO source) with a total dose of 10.12 Gy, which was chosen based on pre-experiments.

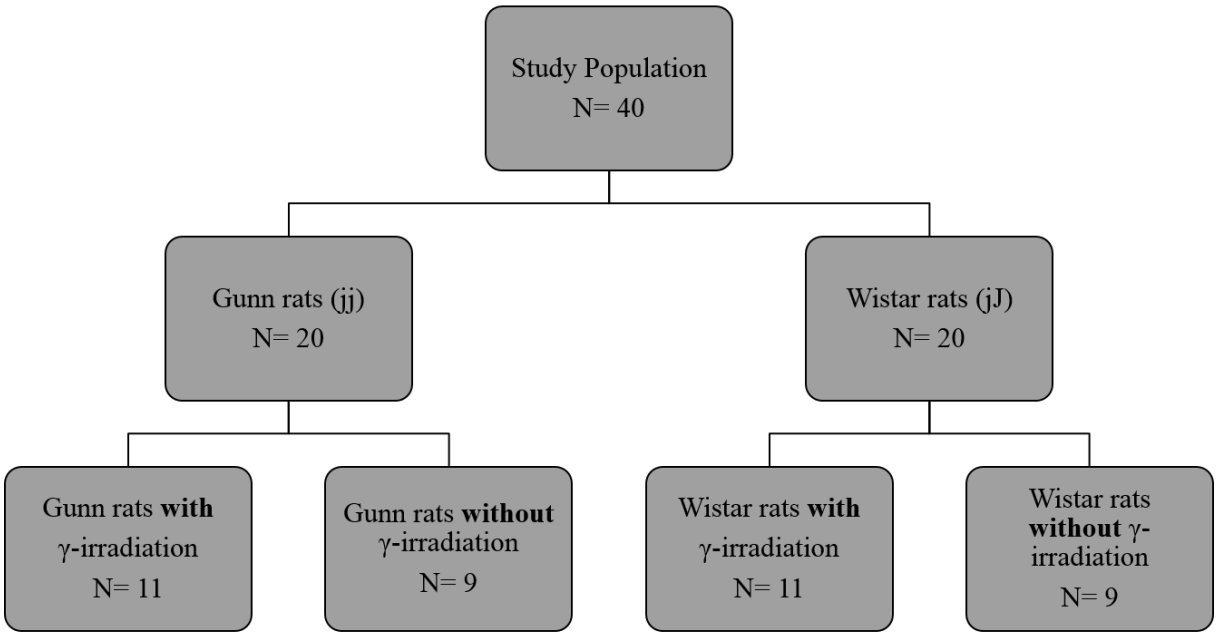

Figure 10: Study design of animal study; Wistar rats as control littermates; jj, homozygous for UGT1A1; jJ, heterozygous for UGT1A1

3.2.1. Sample preparations

Animals were sacrificed immediately after irradiation by decapitation after CO_2 asphyxiation. Subsequently blood, colons and livers were removed. The colon was washed four times with PBS subsequently, cells were collected by gentle scraping and were transferred into cold (4 °C) PBS solution. One gram of liver was transferred into cold buffer (pH7.5) and homogenized (Potter-Elvehjem), centrifuged (800 x g, 4 °C, 10 min. (138)). Blood was collected into sodium-heparinized tubes (Ebewe, Austria) and immediately transferred into tubes containing ficoll (Greiner Bio-One, Austria) for PBMC isolation. Plasma was obtained after centrifugation, aliquotted and stored at -80 °C until further analysis. Isolated PBMCs were washed 2 times with cold PBS and all further treatment was done at 4 °C.

3.3. Bilirubin, anthropometrical measurements and methods of biochemical Parameters (Original article 1, 2, 3, 4)

3.3.1. Unconjugated bilirubin

UCB was determined in serum immediately after centrifugation or within one week, if additional replicates were required at the Department of Nutritional Sciences. A high performance liquid chromatograph (Merck, Hitachi, LaChrom, Austria) equipped with a photo diode array detector (PDA, Shimadzu, Austria) was used to measure unconjugated bilirubin eluted from a Fortis C18 HPLC column (4.6 x 150 mm, 3 µm) with a phenomenex C18 HPLC guard column (4.0 x 3.0 mm) (modified from Brower et al. (139)). An isocratic mobile phase perfused the column and contained 0.1 M di-n-octylamine in methanol:water (95:5; v/v) and glacial acetic acid. UCB was extracted from samples 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 IXα peak was 10.9 minutes. UCB acted as an external standard and had an isomeric purity of >99 % (for 3.3 % IIIα, 92.8 % IXα and 3.9 % XIIIα isomers respectively; 450 nm; Frontier Scientific, United Kingdom).

3.3.2. Anthropometrical measurements

All anthropometrical measurements were performed on participants who were lightly dressed without wearing shoes. Body height (m) was measured with a stadiometer (Seca, Modell 214, Hamburg, Germany) and body weight (kg) using standard analogue scales (Seca Selecta 791, Hamburg, Germany). The body mass index (BMI) was calculated and expressed as kg/m^2.

3.3.3. Blood biochemistry

Liver enzymes γ-GT, AST, ALT, LDH and ALP were analysed on the day of blood sampling using routine diagnostic tests on Olympus 5400 clinical chemistry analysers (Beckman Coulter, Austria) at the General Hospital of Vienna.

3.4. Methods Original Article 1

3.4.1. Micronucleus assay in peripheral blood lymphocytes (CBMN)

Peripheral blood lymphocytes (PBL) were isolated from lithium heparinised blood using ficoll separation tubes (Greiner bio one, Austria). The method of Fenech was conducted for further sample preparation and cell counting (64). A concentration of 10^6 cells/mL was stimulated with phytohemagglutinin (PHA; PAA, Austria) and after 72 h cytochalasin B (Cyt B; Sigma Aldrich, Austria) was added to stop cell division in the binucleated stage. After applying cells on slides and staining (Diff-Qick; Medion Diagnostics, Switzerland) cellular evaluation was conducted using bright-field microscopes with 100-fold magnification (Olympus, Austria). For each subject duplicates were performed and two slides of each duplicate were produced. From the four resulting slides 500 cells/slide (2000 per subject) were counted equally to minimise experimental variation and eliminate scorer bias. With the CBMN-assay chromosomal damage was assessed in lymphocytes as micronucleated cells (MNC_PBL), total micronuclei (MNi_PBL) nuclear buds (NBuds_PBL) and nucleoplasmic bridges (NPBs_PBL, Figure 11). Additionally information on cell death, apoptosis (apo) and necrosis (necr), was collected. The nuclear division index (NDI) was calculated to assess the mitogenic activity of lymphocytes and served as a quality control for the method (77). The CBMN assay was performed at the Department of Nutritional Sciences.

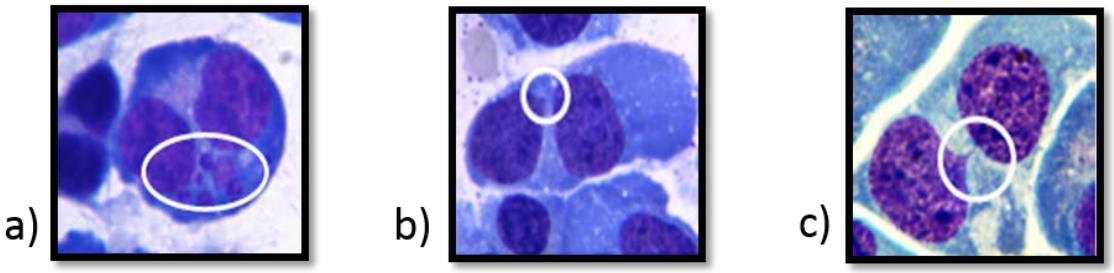

Figure 11: Binucleated PBLs with a) MNi b) NBud c) NB

3.4.2. Micronucleus assay in buccal cells (BMcyt)

Buccal cells were collected from both cheeks of respective subjects, by using a toothbrush and were prepared and scored according to the method of Thomas et al. 2009 (65). A concentration of 80,000 cells/mL was spotted on slides and >2000 cells/slide were counted to ensure accuracy of the results. For staining with Feulgen, cells were placed in beakers with 5.0 M HCl at room temperature for 15 min, rinsed with distilled water (15 min) and subsequently stained with Schiff's reagent (90 min). Cells were scored under bright field with 400-fold magnification using Eclipse E600 microscopes (Nikon, Japan) and then confirmed as positive under fluorescence. MNi were scored in a combination of both basal and differentiated cells according to the criteria defined by Thomas *et al.* (65). The analysis of the slides was carried out by two experienced scorers at the Cancer Research Institute of the Medical University of Vienna; MNi and other nuclear anomalies were recorded after consensus.

The chromosomal damage in differentiated buccal cells (BC) was determined by the BMcyt-assay and cells were scored for micronucleated cells (MNC_BC), total micronuclei (MNi_BC, Figure 12), nuclear buds (NBuds_BC) and binucleated cells (BNC). Furthermore, this method allows the quantification of nuclear anomalies including kariolysis (KL), karyorrhexis and condensed chromatin cells (KR+CC) as well as pycnotic cells (P). Since there were difficulties for discrimination between KR and CC cells, they were considered as one. Furthermore, basal cells were estimated only in a subpopulation of young (<30 years) individuals due to time constraints. These cells are found in the basal cell layer and may assist in estimating the thickness of buccal mucosa membrane and also reflect the turnover rate of buccal cells (140, 141). The measurements were performed at the Vienna Cancer Research Institute.

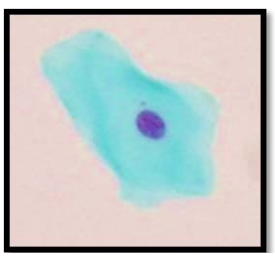

Figure 12: Buccal cell with MNi

3.4.3. Vitamin B12, folic acid and homocysteine

Vitamin B12 was measured in plasma and folic acid in erythrocytes using radioimmunoassays (MP Biomedicals, Germany). Plasma homocysteine was determined using high performance liquid chromatography (HPLC) with a fluorescence detector (emission wavelength: 515 nm; excitation wavelength: 385 nm) on a RP LiChrosphere column (5µm, 125 x 4 mm) (Merck, Hitachi, LaChrom, Austria). Potassium hydrogenphosphate buffer including 4% acetonitrile was used as mobile phase (142). The measurements were performed at the Department of Nutritional Sciences.

3.5. Methods Original Article 2

3.5.1. Single cell gel electrophoresis (SCGE) assay (human cells)

The SCGE assay was performed at the Department of Nutrional Sciences and applied in human (138, 143). Standard conditions (Single-and double DNA strand breaks and apurinic sites) and FPG sensitive sites were measured. DNA damage was induced by H_2O_2 (100 µM, all, ex-vivo). Thirty microliters (approximately $1x10^6$ cells/ml) were 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 (standard conditions, H_2O_2) or triplicates (FPG) were performed. A proportion of the human samples were treated with H_2O_2 solution (100 µM, 5 min., 4 °C). Then, all slides were placed into lysis buffer (pH 10) for a minimum of one hour.

For enzyme treatment 50 µl enzyme-containing buffer (FPG) or enzyme buffer only was pipetted onto the spot and incubated for exactly 30 min. DNA unwinding phase was conducted for 20 min. In the same solution the electrophoresis was performed for 30 min and at 25 V (300 mA) at pH >13 to allow the DNA fragments to migrate.

Evaluation was done after ethidium bromide staining (20 µg/ml). Two replicates (50 cells per slide were evaluated, Figure 13) for each sample were analysed and the mean

(% DNA in tail) was calculated on a fluorescence microscope (Zeiss, Germany) using Komet 5.5 software (Kineting Imaging, UK).

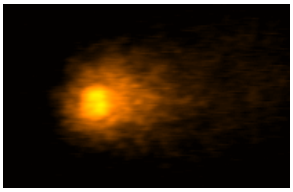

Figure 13: A typical Comets tail

3.5.2. Determination of 8-oxoGuo, 8-oxodG and creatinine

8oxodG and 8oxoGuo, were determined in urine at the Laboratory of Clinical Pharmacology, Rigshospitalet, in Copenhagen. In brief, a chromatographic separation was performed on an Acquity UPLC system (Waters, USA), using an Acquity UPLC BEH Shield RP18 column (1.7 µm, 2.1x100 mm) and a gradient of A: 2.5 mM ammonium acetate (pH 5) and B: Acetonitrile. The MS/MS detection was performed on an API 3000 triple quadrupole mass spectrometer (Sciex, Canada) using electrospray ionisation operated in the positive mode (144). 15N5-8oxoGuo and 15N5-8oxodG were applied as internal standards. Two specific product-ions were measured from each analyte to ensure correct identification and quantification. 8oxodG and 8oxoGuo were normalized against urinary creatinine concentration, which were measured using routine diagnostic tests on Olympus 5400 (Beckman Coulter, Austria) at the General Hospital of Vienna.

3.5.3. Determination of β-carotene, vitamin C and glutathione

Plasma concentrations of β-carotene were determined by reverse-phase HPLC (145). One ml of plasma was mixed with 1 ml ethanol, 100 µl internal standard (Dihydrophyllochinone), 5 ml hexane, mixed properly and centrifuged for 3 min at 3000 rpm. For analysis of carotenoids 500 µl of the hexane phase were dried under nitrogen stream and dissolved in mobile phase (72 % acetonitrile, 10 % methanol and 18 % dichloromethane). The column (LiChrospher 100, RP-18, 5 µm, 250 x 4 mm, Merck, Germany) was thermostatically controlled at 20 °C and a UV-detector at 450 nm was used. Vitamin C (146) and glutathione (147) were determined by spectrophotometry using a UV/VIS spectrometer (Hitachi, Japan). To ensure quality control a control plasma

sample was run throughout the study. Coefficients of variation (CVs) for vitamins and antioxidants were between 2 and 8 %. All measurements were performed at the Department of Nutritional Sciences.

3.5.4. Determination of vitamin B12 and folic acid

See section 3.4.3.

3.5.5. Single cell gel electrophoresis assay (rodent cells)

The SCGE assay in rodents was applied in PBMCs, liver and colonocytes (138, 143) and carried out at the Department of Nutritional Sciences. Single-and double DNA strand breaks and FPG sensitive sites were measured in rodent cells. DNA damage was induced in 22 rats out of 40 (in-vivo) by γ-irradiation (10.12 Gy). Cells were mixed with 1 % low melting point agarose (LMA, Table 1) in PBS at 37°C and applied onto precoated (1 % normal melting point agarose in distilled water) slides. Duplicates (standard conditions) or triplicates (FPG) were performed. Then, all slides were placed into lysis buffer (pH 10) for a minimum of one hour. For enzyme treatment 50µl enzyme-containing buffer (FPG) or enzyme buffer only was pipetted onto the spot and incubated for exactly 30 min. DNA unwinding phase was conducted for 20 min. In the same solution the electrophoresis was performed for 30 min and at 25 V (300 mA) at pH >13 to allow the DNA fragments to migrate. Evaluation was done after ethidium bromide staining (20 µg/ml). Two replicates (50 cells per slide were evaluated) for each sample were analysed and the mean (% DNA in tail) was calculated on a fluorescence microscope (Zeiss, Germany) using Komet 5.5 software (Kineting Imaging, UK).

Table 1: Pipetting scheme: Cell suspension applied onto pre-coated slides

Pipetting scheme	**cellsuspension µl**	**LMA µl**	**Spot µl**
colonocytes	75	135	2x70
hepatocytes	15	150	2x70
lymphocytes	30	140	2x70

3.6. Methods Original Article 3

3.6.1. Determination of apolipoprotein A1, B, lipoprotein (a), triglycerides and HDL (human)

Apolipoprotein A1, apolipoprotein B and lipoprotein(a), serum TG, TC and HDL-cholesterol were analysed on the day of blood sampling using routine diagnostic tests on Olympus 5400 clinical chemistry analysers (Beckman Coulter, Austria) at the General hospital in Vienna.

3.6.2. Determination of LDL-subfractions

The quantitative analysis of 5 lipoprotein families and the lipoprotein subfractions, VLDL, IDL1–3, LDL1, LDL2, LDL3–7, HDL the low-atherogenic lipoprotein profile (IDL3 and LDL1), and the pro-atherogenic lipoprotein profile (VLDL, IDL1-2 and LDL2-7) in serum from a subsample of 36 age and gender matched subjects, was performed using the Lipoprint LDL system (Quantimetrix Corp. CA, USA) (148) at the Comenius University in Bratislava (Slovakia).

3.6.3. Determination of IL-6

Interleukin-6 was determined using high sensitive ELISA from eBioscience. The measurements were performed at the Department of Nutritional Science.

3.6.4. Determination of serum amyloid A (SAA)

SAA was determined at the General hospital of Vienna using the N Latex SAA® Kit from DADE Behring.

3.6.5. Determination of the lipid profile (rodent)

Plasma TC, TG, HDL-C and LDL-C were analysed using routine diagnostic tests on Olympus 5400 clinical chemistry analysers (Beckman Coulter, Austria) at the General hospital of Vienna.

3.7. Methods Original Article 4

3.7.1. Determination of heme

For the determination of the heme peak at 400 nm, the same method as for UCB determination was used (see section 3.3.1.), which was completed at the Department of Nutritional Sciences. Hemin was used as an external standard (>98% purity, rt 6.8 min., Frontier Scientific).

3.7.2. Determination of carboxy hemoglobin

Carboxy hemoglobin was measured using a blood gas analyser ABL 700 (Radiometer) after collecting blood in heparinised syringes and measured immediately at the General Hospital of Vienna.

3.7.3. Determination of cytokines (IL-1β, TNF-α, IL-6)

IL-1β and TNF-α were measured in heparinized plasma and IL-6 in serum using high sensitive-enzyme linked immunoassays (ELISA, eBioscience). All measurements were performed in duplicate and at the Department of Nutrional Sciences.

3.7.4. Determination of C-reactive protein (CRP)

C-reactive protein was analysed using the high sensitive-CRP Latex immune-turbidometric assay on an Olympus 5400 clinical chemistry analyser (Beckman Coulter) at the General Hospital of Vienna.

3.8. Statistical analysis

All statistical tests were completed using SPSS (IBM statistics, Version 17.0). Normal distribution within the data set was tested by the Kolmogorov-Smirnoff Test (KS). For Paper 1 some variables additionally were log10 (UCB and MNi/MNC) transformed and an inverse transformation (1/x) for age was conducted. To determine differences between two groups, an independent sample T-Test (parametric data) or Mann-

Whitney U-Test (non-parametric data) was conducted. Dependent on the homogeneity of variances and normality of data within groups, oneway ANOVA (parametric data) or Kruskal Wallis H-Test (non-parametric data,) was used for multiple group comparisons. Pearson coefficient (parametric data) or Spearman`s rho correlation (non-parametric data) tested the bivariate relationships between two variables. For some evaluations the multiple linear regression analysis was performed to observe the influence of independent variables on the dependent. Data are expressed as mean ± SD and a P-value <0.05 was considered to be significant.

4. Summary of Findings and Discussions

4.1. Original Articles 1 to 4: Comparison of demographic and biochemical parameters between Gilbert`s syndrome subjects and controls[1]

The levels of UCB were significantly higher in the GS group compared to controls. As controls were age and gender matched to GS individuals, there was no difference in age between both groups. However, the BMI was significantly lower in subjects with Gilbert`s syndrome (P= 0.025). Importantly, the comparison of parameters showing liver damage and hemolysis (γ-GT, AST, ALT, LDH and ALP) revealed no difference between the groups and all parameters were within the normal range (Table 2).

Table 2: Demographic and biochemical parameters of controls and individuals with GS

Parameters	Controls (n=38)	Gilbert`s syndrome (n=38)	P-value
Age (years)	31.9 (11.2)	32.3 (11.8)	0.881
BMI (kg/m²)	24.4 (3.22)[a]	22.8 (2.85)	0.025
UCB (µM)	10.3 (3.31)[a]	32.0 (13.6)	<0.001
γ-GT (U/l)	19.5 (7.08)	22.2 (14.4)	0.300
AST (U/l)	24.3 (7.22)	26.7 (7.35)	0.165
ALT (U/l)	21.7 (8.27)	25.5 (14.4)	0.166
LDH (U/l)	157 (23.7)	163 (26.6)	0.315
ALP (U/l)	64.0 (14.9)	70.1 (17.3)	0.105

values as mean (standard deviation), [a]significantly different from the GS group; p< 0.05

The lower BMI in the GS group was a very important finding within this thesis, and confirmed what had already been shown in previous reports (149, 150). This finding might have contributed to the positive health effects in individuals with GS observed within the present study.

[1] In Original Article 3 a higher number of subjects was included, the participants of the present thesis were mixed with further recruited participants to increase the power of the investigation. Therefore, the results of Table 2 (above) are different to the results of Table 1 in Original Article 3.

4.2. Original Article 1: Effects of unconjugated bilirubin on chromosomal damage in individuals with Gilbert`s syndrome measured with the micronucleus cytome assay

In order to measure the chromosomal damage in epithelial cells (bucall cells) and lymphocytes of subjects with Gilbert´s syndrome the micronucleus assay was conducted and compared to age and gender matched controls. Although, the chromosomal changes in GS and control subjects did not differ, the analysis of age sub-groups revealed that the rates of micronucleated buccal cells and buccal NBuds was significantly lower (by 73.3 %, P= 0.014; by 70.9 %, P= 0.008) in the GS group ≥30 years compared to the GS group <30 years of age. The negative correlations of age with frequencies of MNC (r= -0.385, P= 0.017) and NBuds (r= -0.631, P <0.001) in buccal cells of GS individuals referred also to the previous result.

In contrast, age was positively correlated with MNC in lymphocytes (r= 0.553, P <0.001) in the entire study population, what had been commonly reported in other articles (66, 151). The differing effects in both cell types might result from the lower repair capacity and higher turnover rate in buccal cells (40). Furthermore, females had significantly higher frequencies of MNC/MNi (P= 0.019/0.038) in lymphocytes, what had been also previously reported (151, 152).

The increased DNA stability in older GS subjects show that these individuals are protected against the consequence of variation in the genetic material.

4.3. Original Article 2: Anti-genotoxic potential of bilirubin in vivo: Damage to DNA in hyperbilirubinemic human and animal models

To evaluate the damage to DNA/RNA in hyperbilirubinemic subjects versus healthy age and gender matched controls the single cell gel electrophoresis assay and measurements of urinary biomarkers (DNA: 8oxodGuo; RNA: 8oxoGuo) were performed. For detailed information on damaged DNA an animal model using irradiated

vs. non-irradiated hyperbilirubinemic Gunn rats was used and compared to healthy controls (Wistar rats).

No significant difference between Gilbert syndrome and control group regarding levels of damage to DNA (standard conditions, H_2O_2, FPG-sensitive sites, and 8oxod-Guo) and RNA (8oxoGuo) was found. Similarly, antioxidant and vitamin concentrations did not differ between the Gilbert syndrome and the control group. In contrast, the PBMCs of the hyperbilirubinemic Gunn rats had significantly higher strand breaks/apurinic sites than Wistar rats ($P < 0.001$). This could either be due to higher DNA damage itself or might indicate efficient DNA repair (153). The UCB concentrations in some of the Gunn animals were approximately 3 times higher than those in Gilbert syndrome and it is well known that severely elevated UCB concentrations induce toxic effects (154).

Beside the effect of bilirubin in hyperbilirubinemic vs control groups, in the human trial also effects of age, gender and BMI were analysed (Table 3).

In the present study no association between age and damage to DNA/RNA was revealed, although a trend of higher 8oxoGuo levels in the older age group was found ($P = 0.067$). Other studies show inconsistent results concerning age and damage to DNA/RNA (155-157). Within regard to sex, it was also shown that males have higher DNA damage when compared to females (158), which might be due to higher metabolic rates in men (159), and is supported by the fact that men also suffer more often from cancer than women (160). The present study revealed a lower oxidative RNA damage (8oxoGuo, $P < 0.05$) but also a trend for higher strand breaks/apurinic sides ($P = 0.060$) in men. Moreover, higher damage to DNA (assessed by SCGE) with increasing BMI in females was reported (161).

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 (≥25 kg/m^2), leading to the assumption that UCB might be associated with the bodyweight and also with damage to DNA, which, however, has to be further explored.

Table 3: UCB concentration, demographic features and DNA damage of controls and individuals with GS subdivided in groups of age, gender and BMI

Parameters	Age		Gender		BMI	
	<30 years (n=43)	**≥30 years (n=33)**	**Male (n=56)**	**Female (n=20)**	**<25 kg/m² (n=53)**	**≥25 kg/m² (n=23)**
UCB (µmol/l)	19.9 (13.2)	22.7 (16.5)	22.4 (15.5)	17.5 (11.6)	23.6 (16.1)	15.3 (8.55)[h]
Age (years)	24.3 (2.50)	42.2 (10.5)[d]	31.5 (11.4)	33.9 (11.7)	29.7 (9.86)	37.7 (13.0)[h]
BMI (kg/m²)	22.6 (2.83)	24.8 (3.11)[d]	23.8 (2.89)	22.9 (3.71)	21.9 (1.75)	27.4 (1.91)[h]
Standard conditions[a,b]	5.06 (1.31)	5.17 (1.27)	5.27 (1.21)	4.65 (1.31)[f]	4.93 (1.26)	5.51 (1.16)[i]
H_2O_2 sensitive sites[a]	19.6 (4.68)	18.3 (4.55)	19.1 (4.78)	19.0 (4.33)	19.5 (4.77)	18.1 (4.26)
FPG sensitive sites[a]	5.13 (3.08)	4.38 (2.43)	4.98 (2.96)	4.31 (2.40)	4.71 (2.87)	5.02 (2.77)
8oxodG[c]	1.66 (0.60)	1.53 (0.66)	1.58 (0.65)	1.66 (0.56)	1.70 (0.67)	1.38 (0.43)[h]
8oxoGuo[c]	1.75 (0.49)	2.00 (0.66)[e]	1.78 (0.58)	2.10 (0.53)[g]	1.82 (0.55)	1.94 (0.64)

values as mean (standard deviation)
[a]% DNA in Tail
[b] strand breaks and apurinic sites
[c]nmol/mmol creatinine
[d]P<0.05 from age <30 years
[e]P= 0.067 from age <30 years
[f]P= 0.060 from males
[g]P<0.05 from males
[h]P<0.05 from BMI <25 kg/m²
[i]P= 0.064 from BMI <25 kg/m²

4.4. Original Article 3: Protection from age-related increase in lipid biomarkers and inflammation contributes to cardiovascular protection in Gilbert's syndrome

The lipid profile and inflammatory biomarkers were investigated to show whether GS subjects are protected from CVD and compared to normal healthy age and gender matched controls. Additionally, a hyperbilirubinemic rat model (Gunn rats) was explored to support the human data.

Total cholesterol (TC), triglycerides (TG) and LDL-C, LDL subfractions (LDL-1 and LDL-2) and the low-atherogenic parameter IDL-3 were significantly reduced in GS individuals compared to controls (all P <0.05). Furthermore, CVD risk parameters Apo-B and the ratio Apo B/A1 were reduced in GS subjects (P <0.05). The Apo-B/ApoA1 ratio is considered as one of the strongest plasma lipid-associated predictors of CVD risk (162), which indicates the balance between potentially atherogenic and anti-atherogenic

particles (163). Interleukin-6 was significantly lower in GS ($P < 0.05$). A similar trend was shown for SAA ($P = 0.094$).

The animal model supported the results from humans in that the Gunn rats possessed significantly reduced TC, TG and HDL-C ($P < 0.05$) but not LDL-C levels compared to littermate controls. The previous results were more pronounced in female Gunn rats and which also had a lower BMI; the BMI in the human cohort trended ($P = 0.075$) to be significantly lower in GS subjects compared to controls. Negative correlations between UCB and BMI ($r = -0.211$, $P < 0.05$), total cholesterol ($r = -0.245$, $P < 0.01$), LDL-C ($r = -0.243$, $P < 0.01$), triaglycerols ($r = -0.248$, $P < 0.01$), LDL-1 ($r = -0.247$, $P < 0.05$), LDL-2 ($r = -0.272$, $P < 0.05$) and the low atherogenic subfractions (LDL-1 and IDL-3) ($r = -0.276$, $P < 0.05$) support the group differences determined in the human trial. The forward stepwise regression analysis revealed that bilirubin explained 21 % of the variance in LDL-C, 15 % of TC and 4.5 % of LDL-1 in GS subjects. However, bilirubin explained 44.6 % of the variance of total cholesterol, 42.4 % of HDL-C and 28.3 % of triglycerides in Gunn rats. These data could reflect a dose response of circulating bilirubin on lipid metabolism that is species independent. Mechanistically, altered lipid metabolism could reflect a role for either UGT1A1 or UCB in modifying lipid metabolism and cholesterol excretion (164). Furthermore, the impact of bilirubin metabolism on TG and VLDL synthesis might be potentially by down regulating their synthesis (85).

Since lipid parameters, as risk factors for CVD, rise with age, more specific analyses were conducted by dividing the human cohort into two age subgroups (age group 1: <30 years; age group 2: ≥30 years with a range from 30 to 72 years) as shown in Table 4.

For almost all lipid parameters the older controls showed either higher values than older GS subjects or levels tended to be greater. The older control group had significantly higher total cholesterol, triglycerides, LDL-C, LDL-subfractions (except LDL-3), Apo-B and the ratio Apo-B/Apo-A1 than the younger control group ($P < 0.05$). Interestingly, this age related increase in these parameters was not observed between the younger and the older GS group. The older control group had the highest SAA, C-reactive protein and IL-6 concentrations when compared to the other age-subgroups

(older/younger GS subjects and the younger control group; P <0.05). Lower levels of CRP and also IL-6 are related to a lower risk of CVD (165) and diabetes (166) and further emphasize the importance of these biomarkers in contributing to cardiovascular protection in individuals with mildly elevated unconjugated bilirubin levels.

Table 4: The role of age on biomarkers of lipid metabolism and inflammation in GS and control subjects

Parameters	Controls <30 years, n=27	Gilbert`s syndrome <30 years, n=25	Controls ≥30 years, n=32	Gilbert`s syndrome ≥30 years, n=34	ANOVA P-value
Total Cholesterol (mM)	4.97 (0.88)	4.54 (0.91) [b]	5.92 (0.97) [c]	5.30 (1.14) [a]	<0.001
Triglycerides (mM)	1.10 (0.58)	0.95 (0.39)	1.31 (0.53) [c]	1.08 (0.50) [a]	<0.05
HDL (mM)	1.58 (0.35)	1.52 (0.27)	1.63 (0.44)	1.53 (0.38)	n.s.
LDL (mM)	2.76 (0.63)	2.46 (0.71) [b]	3.68 (0.80) [c]	3.08 (0.93) [a]	<0.001
VLDL (mM)	0.63 (0.21)	0.61 (0.18)	0.75 (0.15)	0.62 (0.25) [b]	0.122
LDL-1 (medium LDL) (mM)	1.03 (0.27)	0.93 (0.25)	1.21 (0.30) [c]	0.93 (0.27) [a]	<0.05
LDL-2 (small LDL) (mM)	0.4 (0.40)	0.28 (0.19)	0.51 (0.28) [c]	0.40 (0.24)	<0.05
LDL-3 (very small LDL) (mM)	0.04 (0.04)	0.01 (0.02)	0.03 (0.05)	0.03 (0.04)	n.s.
Pro-atherogenic score*	77.6 (23.1)	70.0 (23.2)	95.1 (20.7) [c]	82.6 (36.3)	<0.05
Low-atherogenic score**	53.9 (12.4)	51.5 (12.7)	63.4 (13.8) [c]	51.4 (14.8) [a]	<0.05
Apolipoprotein A1 (mg/dl)	141 (19)	139 (19)	150 (20)	145 (17)	n.s.
Apolipoprotein B (mg/dl)	83.6 (18.6)	79.4 (19.2)	98.8 (18.4) [c]	89.6 (26.4) [b]	<0.05
Ratio Apo-B/Apo-A1	0.60 (0.15)	0.58 (0.18)	0.69 (0.15) [c]	0.61 (0.21) [a]	<0.05
Lipoprotein (a) (mg/dl)	40.0 (38.6)	29.2 (28.2)	34.9 (31.9)	46.8 (55.9)	n.s.
SAA (mg/l)	3.47 (1.21)	3.42 (0.74)	4.75 (2.11) [c]	3.61 (0.87) [a]	<0.05
C-reactive Protein (mg/dl)	0.14 (0.20)	0.12 (0.17)	0.16 (0.15) [c]	0.14 (0.14)	n.s.
IL-6 (pg/ml)	0.90 (0.71)	0.78 (0.43)	1.59 (1.36) [c]	0.72 (0.32) [a]	n.s.

[a]P <0.05 to control of the age group (young or old); [b]P for trend < 0.1 to control of the age group (young or old); [c] P <0.05 to younger GS and control; *pro-atherogenic score: VLDL, IDL1-2 and LDL2-7; **low-atherogenic score: IDL-3 and LDL-1.

The presented data show that elevated circulating bilirubin, particularly the benign condition of Gilbert`s Syndrome, is associated with lower lipid biomarker lower inflammation and trends to a decreased BMI. Furthermore, it is shown that older subjects, who are generally at greater disease risk for CVD, are likely to benefit more from a mild congenital hyperbilirubinemia. These data are strongly supported by serum lipid analysis in Gunn rats, with more pronounced effects in the female animals.

4.5. Original Article 4: Heme catabolism: a novel modulator of inflammation in Gilbert's syndrome

It is known that bilirubin levels are negatively correlated to the prevalence and mortality associated with inflammatory conditions such as CAD, atherosclerosis and myocardial infarction (119, 167, 168). Also, GS subjects possess a reduced risk of CVD and inflammatory bowel disease (121, 124). Consequently, revealing the association of catabolites of heme metabolism (heme, CO, iron and UCB) with changes in inflammatory parameters (IL-6, CRP, IL-1 β and TNF- α) was the center of investigation.

Subjects with GS had higher levels of iron (*P* <0.001), CO-Hb (*P*= 0.009), hs-IL-1β (*P*= 0.003) and lower levels of IL-6 (*P*= 0.027) compared to the control group (Table 5). In this context, bilirubin was positively correlated with iron (r= 0.562, P <0.05), CO-Hb (r= 0.231, P <0.05), heme (r= 0.224, P <0.05), hs-IL-1β (r= 0.314, P <0.05) and hs-TNF-α (r= 0.242, P <0.05). Furthermore, UCB was negatively correlated with the BMI (r= -0.290, P <0.05), hs-IL-6 (r= -0.228, n.s.) and hs-CRP (r= -0.222, n.s.).

Table 5: Biochemical and inflammatory parameters of controls and GS subjects

Parameters	Controls (n=38)	Gilbert`s syndrome (n=38)	P-value
Heme (µM)	0.51 (0.44)	0.64 (0.47)	0.242
Fe (µM)	22.4 (9.15)[a]	32.3 (8.32)	<0.001
CO-Hb (%)	0.91 (0.23)[a]	1.06 (0.25)	0.009
hs-IL-1β (pg/ml)	2.07 (0.15)[a]	2.21 (0.24)	0.003
hs-TNF-α (pg/ml)	2.65 (0.35)	2.54 (0.34)	0.171
hs-IL-6 (pg/ml)	1.19 (1.07)[a]	0.75 (0.39)	0.027
hs-CRP (mg/dl)	0.16 (0.21)	0.09 (0.08)	0.413

values as mean (standard deviation), [a]significantly different from the GS group; P< 0.05,

Subsequently, multiple linear regression analysis was conducted to explore the ability of independent variables to explain inflammatory endpoints (Table 6). Only bilirubin could explain variations in IL-1β concentrations. Although, IL-1β is thought to be a typical

proinflammatory molecule, it might also prime neighboring cells to increase their mitochondrial ROS production to resist pathogen invasion (110).

Table 6: Multiple regression analysis for cytokines as dependent variables

Dependent variables	Independent variables	R^2 (%)[a]	β[b]	P-value
IL-1β[c]	UCB	14.5	0.006	0.001
TNF-α[d]	age	18.8	0.014	<0.001
IL-6[e]	CRP	21.4	2.009	0.003
	+ iron	27.1	-0.026	0.026
CRP*[f]	BMI	21.4	0.085	<0.001
	hs-IL-6	28.0	0.016	0.017

*variables transformed
[a]R^2 adjusted correlation coefficient and present cumulative explanation of dependent variable in % (for more than 1 variable from top to bottom)
[b]unstandardized B
[c]excluded variables: age, sex, BMI, CO-Hb, Fe, heme, hs-TNF-α,
[d]excluded variables: sex, BMI, UCB, CO-Hb, Fe, heme, hs-IL-1β
[e]excluded variables: age, sex, BMI, UCB, CO-Hb, Fe, heme
[f]excluded variables: age, sex, UCB, CO-Hb, Fe, heme

Figure 11 shows the hypothesized involvement of heme catabolites in a positive feedback loop on HO activity. First, unconjugated bilirubin (UCB) induces a mild hemolytic effect, leading to heme degradation by HO and increased HO metabolite production (biliverdin/UCB, CO, iron). Bilirubin may also directly stimulate HO-1 transcription. Second, the release of IL-1β followed by elevated HO (end-) products, which contribute to a lower BMI and IL-6 concentrations as bilirubin concentrations increase and may act indirectly on CRP production, by reducing IL-6 concentrations. Dashed lines indicate an effect specifically evident in Gilbert's Syndrome.

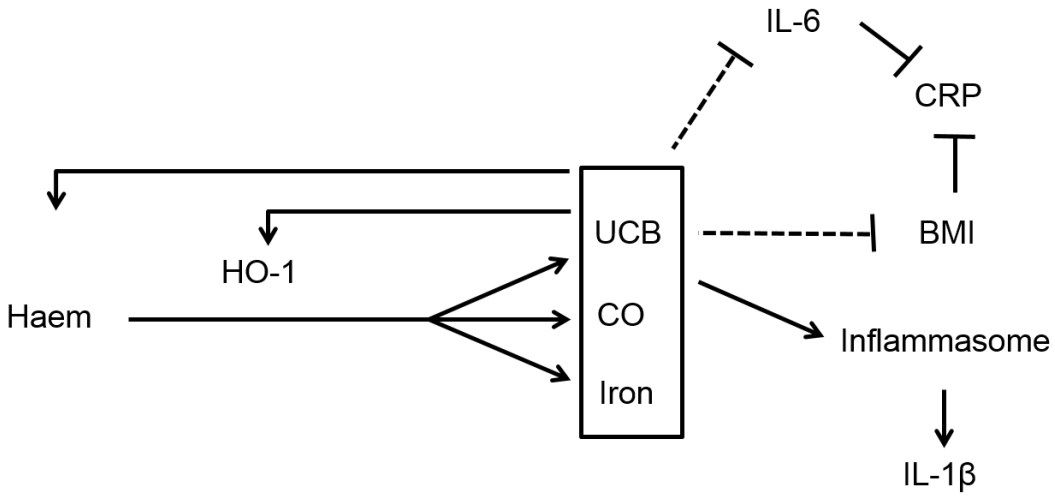

Figure 14: Hypothesized mechanism of bilirubin`s involvement in a positive feedback loop on HO activity

In summary, these results are the first to suggest a potential effect of bilirubin on the production of anti-inflammatory heme catabolites via a positive feedback loop of HO induction (Figure 14) *in vivo*, mediated by bilirubin induced hemolysis. This effect could further amplify the anti-inflammatory and lipid lowering effects of CO and bilirubin, particularly in GS. The novel finding that IL-6 and BMI were reduced in GS, provides mechanistic insight into reduced CRP in this cohort. Finally, elevated IL-1β concentrations in GS and a positive relationship between bilirubin and IL-1β, were unexpected findings, which require further investigation.

5. Conclusions and Future Aspects

Taken together, this study did not reveal a protective effect of chronically elevated UCB on biomarkers of DNA/RNA oxidation and DNA instability. However, older subjects reflect decreased chromosomal anomalies which indicate a protection against the consequence of variation in the genetic material. Other plasma antioxidants were not associated with oxidative DNA/RNA damage (except for vitamin B12) and with chromosomal variation.

On the contrary, it was clearly shown that the benign condition of Gilbert`s syndrome is accompanied with reduced levels of lipid and inflammation associated biomarkers. This outcome is supported by the results of the animal model with a stronger effect in the female rats. The presented positive feedback loop of HO and decreased IL-6 levels contributes to a lower inflammation status in hyperbilirubinemic subjects. Combined, these novel observations provide further mechanistic insight into the reduced prevalence of NCD related mortality in GS individuals. Furthermore, this outcome could assist in developing strategies to mildly increase circulating bilirubin concentrations for protection against NCDs.

Importantly, hyperbilirubinemic subjects had a lower BMI which might indicate an indirect effect of UCB in health promotion. Older subjects, who are generally at greater disease risk for chronic disease, are likely to benefit more from a mild congenital hyperbilirubinemia.

However, further investigations on bilirubin’s anti-oxidative and anti-genotoxic effects are of high importance to reveal mechanistic explanations for the existing association between bilirubin and NCDs. Therefore, studies with increased numbers of subjects should be investigated. Particularly attention should be laid on the age and the BMI of participants.

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9. Further Publications

Molzer C, Huber H, Diem K, Wallner M, Bulmer AC, Wagner KH. **Extracellular and intracellular anti-mutagenic effects of bile pigments in the Salmonella typhimurium reverse mutation assay.** Toxicol In Vitro. 2013;27:433-7.

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Molzer C, Pfleger B, Putz E, Rossmann A, Schwarz U, Wallner M, Bulmer AC, Wagner KH. **In vitro DNA-damaging effects of intestinal and related tetrapyrroles in human cancer cells.** Exp Cell Res. 2013;319:536-45.

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Mullner E, Brath H, Pleifer S, Schiermayr C, Baierl A, Wallner M, Fastian T, Millner Y, Paller K, Henriksen T, Poulsen HE, Forster E, Wagner KH. **Vegetables and PUFA-rich plant oil reduce DNA strand breaks in individuals with type 2 diabetes.** Mol Nutr Food Res. 2013;57:328-38.

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Mullner, E., Brath, H., Nersesyan, A., Nitz, M., Petschnig, A., Wallner, M., Knasmuller, S., Wagner, K.-H. 2013. **Nuclear anomalies in exfoliated buccal cells in healthy and diabetic individuals and the impact of a dietary intervention.** (accepted for publication, Mutagenesis)

Oral/poster Presentations

Anti-genotoxic potential of bile pigments – evidence from the Vienna studies
Trieste Yellow Retreat 2011 (Trieste, Italy), June 6 - 7 (oral presentation).

The physiological potential of Bilirubin: A possible antigenotoxic effect?
ÖGE Annual Meeting 2011- Nutrition in health promotion and disease prevention (Vienna, Austria), October 19 – 21 (poster presentation)

Bile pigments protect the DNA in oral cells
ÖGE Annual Meeting 2011- Nutrition in health promotion and disease prevention (Vienna, Austria), October 19 – 21 (poster presentation)

The physiological importance of bilirubin: A possible chemopreventative effect?
ISFE Symposium 2011 – Diet Quality (Vienna, Austria), December 1 - 2 (poster presentation)

The physiological importance of bilirubin: A possible chemopreventative effect?
ASTOX/ANTIOX Symposium 2011 (Vienna, Austria), November 24 (poster presentation)

Curriculum Vitae

Personal Information:

Name:	Marlies Wallner
Date and Place of Birth:	July 22nd, 1983 in Austria
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Education

June 2003	Graduation Highschool (Graz, Austria)
2003-2009	Study of Nutritional Sciences, University of Vienna, Austria Title of diploma thesis: *"Vergleich unterschiedlicher Extraktionsmethoden bei der Bestimmung von Vitamin E und Carotinoiden in Lebensmitteln"* Supervisor: Univ. Prof. Dr. Karl-Heinz Wagner
June 2009	Graduated as Mag. rer. nat.
Since 2009	Doctoral studies of nutritional sciences at the Department of Nutritional Sciences, University of Vienna, Austria Title of doctoral thesis*: "Anti-oxidative and anti-genotoxic potential of unconjugated Bilirubin"* Supervisor: Univ. Prof. Dr. Karl-Heinz Wagner

Work Experience

2009-2010	Tutorial assistance at the Department of Nutritional Sciences, *"Übungen zur Ernährung des Menschen I"*, University of Vienna, Austria
2009-2012	Scientific employee at the Department of Nutritional Sciences, University of Vienna, Austria
From 2010	Lectureship at the Department of Nutritional Sciences, *"Übungen zur Immunologie"* University of Vienna, Austria

Awards

2008/2009	Mobilitätsstipendium “Erasmus”, ÖAD, Vienna, Austria
2009	Award for diploma thesis, Dr. Maria Schaumayer-Stiftung, Vienna, Austria
2010	Förderstipendium, University of Vienna, Austria

Internships (national and international)

Jul-Aug 2007	Laboratory-internship (Pharmaceutical Company, Roche Diagnostics Graz GmbH), Graz, Austria
Oct 2007	Laboratory-internship (Institute for Hygiene, Food Hygiene), Vienna, Austria
Jan 2008	Internship, European nutrition day (Austrian Society for Clinical nutrition), Vienna, Austria
Sep 2008- Mar 2009	Laboratory-internship (Food production Company, Hengstenberg GmbH&Co), Esslingen, Germany
Sep-Dec 2011	Research visit at the Heart Foundation Research Centre, Griffith University, Australia

Further Education

Feb 2010	Flow cytometry seminar, General Hospital of Vienna (AKH), Austria
Mar-Jun 2010	English training (English B2-Phase 2)
Dec 2010	Statistical training, *“Einführung in das Statistikprogramm SPSS”*
Jan 2011	Statistical training, *“SPSS-Fortsetzung”*
Apr 2011	Scientific writing in english, *“Der rote Faden in English academic writing and how to spin it”*

Professional Qualifications

- Analytical skills: HPLC (vitamin and porphyrrin assays), PBMCs in cell culture, Flow cytometry, SCGE assay, CBMN assay, Fluorescence and UV photometry (ORAC, FRAP), ELISA
- Designing of studies, experience with ethical commissions, statistics
- PC skills: Word, Excel Power Point, SPSS, Sigma stat
- Languages: German (native), English (fluent), Spanish (basics)
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- Mentoring of diploma- and master students

1

Original Article 1

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Effects of unconjugated bilirubin on chromosomal damage in individuals with Gilbert`s syndrome measured with the micronucleus cytome assay

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Circulating unconjugated bilirubin (UCB) has been reported to protect against lung and colorectal cancer. The present study aimed to explore, for the first time, whether mildly elevated circulating UCB, as found in Gilbert`s syndrome (GS), is associated with changes of DNA damage. A random 76 individuals, matched for age and gender, were recruited from the general population and allocated into the GS group (UCB ≥17.1 µM; *n* = 38) or control group (UCB <17.1 µM; *n* = 38). Chromosomal and cytological changes were determined in lymphocytes and buccal cells using the cytokinesis-block micronucleus cytome assay (CBMN) and buccal micronucleus cytome assay (BMcyt). No significant differences were found between GS subjects and the control group in the CBMN and BMcyt determined endpoints. Subsequently, when age dependency of effects were analysed, lower formation of buccal micronucleated cells (by 73.3%) and buccal nuclear buds (by 70.9%) in the GS subgroup ≥30 years were found, compared to the GS subgroup <30 years. These findings suggest DNA protection in epithelial tissue of older individuals with GS.

Key words: BMcyt, buccal cells, CBMN, chromosomal damage, Gilbert`s Syndrome, lymphocytes, Micronucleus assay, unconjugated bilirubin

Introduction

Gilbert's syndrome (GS) is a benign condition resulting in moderately elevated unconjugated bilirubin (UCB) levels (≥17.1 µM). GS is usually associated with the occurrence of an autosomal recessive hereditary polymorphism affecting 3–13% of the general population (~12.4% men, ~4.8% women) (1–3). The polymorphism leads to a reduced enzyme function of uridine diphosphate glucuronosyl transferase (UGT1A1), which conjugates bilirubin for its excretion into the bile (2).

The protective effect of UCB on epithelial cancer development is reported repeatedly in the literature. For example, the prevalence and incidence of lung and colon cancers were found to be reduced with increasing circulating UCB concentrations (4–6). Recently, a protective role of the *UGT1A1*28* allele on colorectal cancer (CRC) was detected—CRC patients had lower serum bilirubin levels than controls (7). Potential protective mechanisms involve the antioxidant properties of bilirubin and inhibition of oxidative stress (8–10). Reactive oxygen species (ROS) have DNA damaging properties which suggest to play a role in development of cancer and other diseases (11).

The aim of the present study was to investigate the DNA damage and cytotoxicity in individuals with mildly increased UCB levels. It was hypothesised that endpoint markers for genomic instability would be decreased in GS.

The cytokinesis-block micronucleus cytome assay (12) and the buccal micronucleus cytome assay (13) are well established, widely used and sensitive methods for estimation of chromosomal damage. It has been shown earlier that micronuclei in lymphocytes are reliable markers for cancer rate and it is also well documented that a relationship exists between increased micronuclei (MNi) frequency and the incidence of lung cancer (14). More recent findings suggest that MNi in buccal cells are also valid markers for human cancer risks (15). More than 90% of tumours develop in epithelial tissue (16). Therefore, it was emphasised that the BMcyt is more suitable for human biomonitoring than lymphocytes, which were widely used in the past. In addition to MNi, which reflect structural and chromosomal aberrations (14); other DNA instabilities such as nuclear buds (NBuds), nucleoplasmic bridges (NPBs) and binucleated cells (BN) were scored. In order to collect information about cytotoxicity apoptosis (apo), necrosis (necr), karyolysis (KL), karyorrhexis (KR), condensed chromatin (CC) and pycnotic (P) cells were determined.

At present, no data are available that show the association between bilirubin and cancer related biomarkers in buccal cells. One study investigated the relationship between *UGT1A1* polymorphisms and MNi in human lymphocytes. However, UCB levels were not determined and therefore no firm conclusion could be drawn regarding the importance of bilirubin *per se* (17).

Materials and methods

Study design-subjects

For this study, 104 subjects were recruited from the general population. Of these, 76 subjects met the inclusion criteria as they possessed normal liver function, absence of any underlying 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 (ALP), haemoglobin and hematocrit were measured during the screening examination. Subjects with liver, heart or kidney disease, hemolysis, diabetes, cholelithiasis, organ transplants, history of CVD, cancer, smoking (>1cig/day), alcohol consumption (>7 standard drinks/week), excessive physical activity (>10 h/week), any medication that might affect the liver metabolism and vitamin supplementation (4 weeks prior to the first blood sampling) were exclusion criteria. Allocation to the GS group was determined by a fasting serum UCB concentration of ≥17.1 µM measured by high performance liquid chromatography (HPLC). Subjects were age and gender matched (28 men, 10 women) in each group (total 76 subjects). This study was approved by the ethical committee of the Medical University of Vienna and the General Hospital of Vienna (# 274/2010) and performed according to the Declaration of Helsinki.

Sample preparation

A 24-h fasting (400 kcal food restriction) blood sample was collected into lithium heparin and serum vacutainers. Samples were stored on ice in the dark until

further analysis. Blood collection tubes were centrifuged (15 min, 3000 rpm, 4°C) and serum or plasma was aliquotted, used fresh or stored at −80°C for further analysis. Erythrocytes from lithium heparin vacutainers were washed three times with isotonic phosphate buffer, aliquotted and stored at −80°C.

Blood biochemistry

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

Vitamin B12, folic acid and homocysteine

Vitamin B12 was measured in plasma and folic acid in erythrocytes using radioimmunoassays (MP Biomedicals, Germany). Plasma homocysteine was determined using HPLC, with a fluorescence detector (emission wavelength: 515 nm; excitation wavelength: 385 nm) on a RP LiChrosphere column (5µm, 125 × 4 mm) (Merck, Hitachi, LaChrom, Austria). Potassium hydrogenphosphate buffer including 4% acetonitrile was used as mobile phase (18).

Unconjugated bilirubin

UCB was determined in serum immediately after centrifugation or within one week, if replicates were required. HPLC (Merck, Hitachi, LaChrom, Austria) equipped with a photo diode array detector (PDA, Shimadzu, Austria) was used to measure unconjugated bilirubin 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) (modified from Brower *et al.* (19)). An isocratic mobile phase perfused the column and contained 0.1 M di-*n*-octylamine in methanol:water (95:5; v/v) and glacial acetic acid. UCB was extracted from serum by mixing 40 µl with 160 µl mobile phase, after centrifugation 50 µl of the supernatant was injected at a flow of 1 mL/min. Retention time of the main IXα peak was 10.9 min. Unconjugated bilirubin acted as an external control and possessed an isomeric purity of >99% (for 3.3% IIIα, 92.8% IXα and 3.9% XIIIα isomers respectively; 450 nm; Frontier Scientific, UK).

CBMN _PBL

Peripheral blood lymphocytes (PBL) were isolated from lithium heparinised blood via ficoll separation tubes (Greiner bio one, Austria). The method of Fenech was conducted for further sample preparation and cell counting (12). A concentration of 10^6 cells/ml was stimulated with phytohemagglutinin (PHA; PAA, Austria) and after 72 h cytochalasin B (Cyt B; Sigma Aldrich, Austria) was added to halt cell division in the binucleated stage. After applying cells on slides and staining (Diff-Qick; Medion Diagnostics, Switzerland) cellular evaluation was conducted using bright-field microscopes with 100-fold magnification (Olympus, Austria). For each subject, duplicates were performed and two slides of each duplicate were produced. From the four resulting slides 500 cells/slide (2000 per subject) were counted equally to minimise experimental variation and eliminate scorer bias.

BMcyt_BC

Buccal cells were obtained by rubbing a toothbrush on the insides of both cheeks of subjects and were prepared and scored according to the method of Thomas *et al.* 2009 (13). A concentration of 80 000 cells/mL was applied on slides and >2000 cells/slide were counted to ensure accuracy of the results. For staining with Feulgen, cells were placed in beakers with 5.0 M HCl at room temperature for 15 min, rinsed with distilled water (15 min) and subsequently stained with Schiff's reagent (90 min). Cells were scored under bright field with 400-fold magnification using Eclipse E600 microscopes (Nikon, Japan) and then confirmed as positive under fluorescence. MNi were scored in a combination of both basal and differentiated cells according to the criteria defined by Thomas et al. (13). The analysis of the slides was carried out by two experienced scorers; MNi and other nuclear anomalies were recorded after consensus.

With the CBMN-assay, chromosomal damage was assessed in lymphocytes as micronucleated cells (MNC_PBL), total micronuclei (MNi_PBL) nuclear buds (NBuds_PBL) and nucleoplasmic bridges (NPBs_PBL). Additionally, information on cell death, apoptosis (apo) and necrosis (necr) was collected. The nuclear division index (NDI) was calculated to assess the mitogenic activity of lymphocytes and served as a quality control for the method (20). The chromosomal damage in differentiated buccal cells (BC) was determined by the BMcyt-assay and cells were scored for micronucleated cells (MNC_BC), total micronuclei (MNi_BC), nuclear buds (NBuds_BC) and binucleated cells (BNC). Furthermore, this method allowed the quantification of nuclear anomalies including kariolysis (KL), karyorrhexis and condensed chromatin cells (KR+CC) as well as pycnotic cells (P). Since there were difficulties for discrimination between KR and CC cells, they were considered together.

Table I. Statistical evaluation of non-parametric data

Statistical test	Group	Endpoint analysed
Mann-Whitney U-Test and Spearman rho Correlation	**GS/Control**	Apo, NPB_PBLs, MNC/MNi_BC, NBuds_BC, Folic acid
	Age (<, ≥ 30 years)	NPB_PBLs, NBuds_PBLs, MNC/MNi_BC, NBuds_BC, P
	Gender (male, female)	NPB_PBLs, NBuds_PBLs, MNC/MNi_BC, NBuds_BC, KL, P, Folic acid, Vitamin B12
	BMI (<, ≥ 25 kg/m²)	NPB_PBLs, NBuds_PBLs, MNC/MNi_BC, NBuds_BC, P, Folic acid,
Kruskal Wallis H-Test	**GS** (<, ≥ 30 years) **Control** (<, ≥ 30 years)	NPB_PBLs, NBuds_PBLs, MNC/MNi_BC, NBuds_BC, KL, Folic acid,
Spearman rho Correlation	**Total studypopulation** **Age** (≥ 30 years)	MNC_BC, NBuds_BC,MNC_BC, NBuds_BC

(KS-Test P <0.05)

Table II. Demographic features, biochemical parameters and nuclear anomalies in lymphocytes and buccal cells of controls and individuals with GS

	Parameters	Controls (n = 38)	Gilbert`s syndrome(n = 38)	p-value
	Age (years)	31.9 (11.2)	32.3 (11.8)	0.881
	BMI (kg/m²)	24.4 (3.22)[a]	22.8 (2.85)	0.025
	UCB (µM)	10.3 (3.31)[a]	32.0 (13.6)	<0.001
	γ-GT (U/l)	19.5 (7.08)	22.2 (14.4)	0.300
	AST (U/l)	24.3 (7.22)	26.7 (7.35)	0.165
	ALT (U/l)	21.7 (8.27)	25.5 (14.4)	0.166
	LDH (U/l)	157 (23.7)	163 (26.6)	0.315
	ALP (U/l)	64.0 (14.9)	70.1 (17.3)	0.105
	Folic acid (ng/ml)	163 (173)	142 (116)	0.608
	Vitamin B12 (ng/l)	280 (125)	283 (121)	0.900
	Homocysteine (µM)	14.8 (11.8)	12.2 (4.58)	0.783
CBMN	MNC_PBL	10.6 (10.5)	11.0 (7.3)	0.478
	MNi_PBL	12.2 (12.9)	12.3 (9.0)	0.532
	NPBs_PBL	1.08 (1.19)	1.13 (1.42)	0.660
	NBuds_PBL	2.41 (1.64)[b]	3.43 (2.89)	0.065
	Apo (%)	0.84 (0.47)	0.70 (0.35)	0.201
	Necr (%)	0.60 (0.45)	0.61 (0.42)	0.951
	NDI	2.08 (0.22)	2.09 (0.17)	0.709
BMCyt	MNC_BC	0.22 (0.32)	0.31 (0.39)	0.360
	MNi_BC	0.31 (0.45)	0.35 (0.44)	0.631
	NBuds_BC	0.59 (0.63)	0.56 (0.49)	0.992
	BNC	12.1 (4.25)	12.0 (5.26)	0.933
	KR+CC	9.51 (5.73)	9.78 (5.66)	0.834
	KL	25.7 (17.4)	33.0 (28.1)	0.184
	P	1.05 (0.86)[b]	1.41 (0.93)	0.086
	Basal cells[c]	14.1 (6.07)[b]	9.75 (4.81)	0.070

CBMN values are per 1000 BN cells and BMCyt values are per 1000 differentiated cells; values as mean (standard deviation), [a]significantly different from the GS group; $P < 0.05$, [b]differences for trend from the GS group $P < 0.1$, [c]cohort size: GS n = 12, C n = 11

Table III. UCB concentration, demographic features and nuclear anomalies in lymphocytes and buccal cells of controls and individuals with GS in different groups of age, gender and BMI

	Parameters[a]	Age		Gender		BMI	
		<30 years (n = 43)	≥30 years (n = 33)	Male (n = 56)	Female (n = 20)	<25 kg/m² (n = 53)	≥25 kg/m² (n = 23)
	UCB (μmol/l)	19.9 (13.2)	22.7 (16.5)	22.4 (15.5)	17.5 (11.6)	23.6 (16.1)	15.3 (8.55)[f]
	Age (years)	24.3 (2.50)	42.2 (10.5)[b]	31.5 (11.4)	33.9 (11.7)	29.7 (9.86)	37.7 (13.0)[f]
	BMI (kg/m²)	22.6 (2.83)	24.8 (3.11)[c]	23.8 (2.89)	22.9 (3.71)	21.9 (1.75)	27.4 (1.91)[e]
CBMN	MNC_PBL	6.70 (3.12)	13.6 (7.53)[b]	8.82 (5.98)	12.8 (7.22)[d]	8.78 (5.66)	11.8 (7.65)
	MNi_PBL	7.15 (3.29)	16.1 (10.4)[b]	10.2 (8.32)	14.1 (8.71)[d]	9.49 (6.62)	14.6 (11.1)
	NPBs_PBL	0.79 (0.70)	1.18 (1.26)	0.93 (1.06)	1.11 (0.83)	0.96 (1.07)	1.00 (0.87)
	NBuds_PBL	2.27 (1.75)	3.70 (2.83)[c]	2.85 (2.35)	3.11 (2.56)	2.84 (2.58)	3.09 (1.93)
BMCyt	MNC_BC	0.34 (0.39)	0.17 (0.27)[c]	0.29 (0.36)	0.21 (0.35)	0.25 (0.36)	0.30 (0.36)
	MNi_BC	0.42 (0.49)	0.21 (0.33)	0.34 (0.42)	0.30 (0.50)	0.30 (0.44)	0.38 (0.45)
	NBuds_BC	0.70 (0.54)	0.41 (0.55)[c]	0.61 (0.57)	0.48 (0.55)	0.54 (0.56)	0.64 (0.56)
	BNC_BC	13.1 (4.62)	10.6 (4.62)[c]	12.2 (4.72)	11.6 (4.94)	12.6 (4.74)	10.7 (4.63)

[a]CBMN values are per 1000 BN and BMCyt values are per 1000 differentiated cells; values as mean (standard deviation); [b]$P < 0.001$ from age < 30 years; [c]$P < 0.05$ from age < 30 years; [d]$P < 0.05$ from males; [e]$P < 0.001$ from BMI < 25 kg/m²; [f]$P < 0.05$ from BMI < 25 kg/m²

Furthermore, basal cells were estimated only in a subpopulation of young (<30 years) individuals, due to time constraints. These cells are found in the basal cell layer and may assist in estimating the thickness of buccal mucosa membrane and also in the turnover rate of buccal cells (21,22).

Statistical analysis

All statistical tests were completed using SPSS (IBM statistics, Version 17.0). Normal distribution within the data set was tested by the Kolmogorov–Smirnoff Test (KS). Therefore, some variables were $\log_{10}$ (UCB and MNi/MNC) transformed and an inverse transformation (1/×) for age was conducted. To determine the differences between two groups, an independent sample T-Test (parametric data) or Mann–Whitney U-Test (non-parametric data, Table I) was conducted. Depending on the homogeneity of variances and normality of data within groups, oneway ANOVA (parametric data) or Kruskal Wallis H-Test (non-parametric data, Table I) was used for multiple group comparisons. Pearson coefficient (parametric data) or Spearman rho correlation (non-parametric data, Table I) tested the bivariate relationships between independent and dependent variables. Data are expressed as mean ± SD and a P-value <0.05 was considered to be significant.

Results

UCB concentrations were significantly higher in GS subjects (P <0.001) as compared to controls. Vitamin B12, folic acid and homocysteine did not significantly differ between both groups. It is notable that after age and gender matching, GS subjects had a significantly lower BMI than respective controls (P = 0.025; Table II).

The results which were obtained in CBMN and BMcyt are summarised in the lower part of Table II. It can be seen that no significant differences between GS and controls for all endpoints of lymphocyte and buccal cell chromosomal damage were found. However, a trend was observed for increased NBuds (P = 0.065) in lymphocytes and pycnotic cells from the buccal mucosa in GS subjects (P = 0.086; Table II).

No correlations between UCB and endpoints were found when considering the total study population, although two significant correlations were detected within the GS group. UCB was negatively correlated to MNC_BC (r = −0.380, P = 0.042) and to pycnotic cells (r = −0.394, P = 0.034), whereas no significant correlations were found within the control group.

Furthermore, exploratory analyses were also taken into consideration, as we investigated subjects with a broad age range and recruited both sexes. Surprisingly, the BMI in the GS group was lower and so we, as an ancillary, explored effects of BMI on different nuclear parameters. The results are summarised in Table III. Age did not influence the UCB levels, while the BMI was significantly higher in individuals ≥30 years of age (P = 0.003). Age group 2 (≥30 years) also had significantly higher MNC/MNi (P <0.001) values and NBuds in PBLs (P = 0.019). No gender specific differences were observed for UCB, age and BMI. However, females had significantly higher frequencies of MNC/MNi (P = 0.019/0.038) in lymphocytes. Overweight individuals had significantly lower levels of UCB (P = 0.042) and were older than participants with a BMI < 25 kg/m² (P = 0.005). Furthermore, the overweight subjects trended to have more MNC lymphocytes (P = 0.055; Table III).

A positive association between frequency of MNC in lymphocytes and age was found in the total study population, showing elevated chromosomal instability in lymphocytes in older subjects (r = 0.553, P <0.001), whereas only a weak negative correlation was found for MNC in buccal cells and age (r = −0.222, P = 0.054). After performing individual analysis within GS and control groups the correlation consisted between MNC_PBL and age (GS: r = 0.619, P <0.001; C: r = 0.487, P <0.001). Correlations of age with frequencies of MNC and NBuds in buccal cells differed between the GS and the control group, since there was a negative correlation for individuals with GS (MNC: r = −0.385, P = 0.017; NBuds: r = −0.631, P = <0.001) and no significant correlation for control subjects.

Since these results indicated an age impact on chromosomal damage, a more specific analysis was conducted in order to evaluate differences of nuclear anomalies. Therefore, the GS and control groups were divided into two age subgroups (<30 years; ≥30 years). It can be seen that the MNC rates were significantly lower (by 73.3%, P = 0.014) in the GS subjects ≥ 30 years as compared to those seen in the GS individuals aged <30 years, while no such difference was seen in both age control subgroups. Likewise, we found also lower frequencies of buccal NBuds in the older GS age group (by 70.9%, P = 0.008). In all other markers, no differences were found (Table IV).

Discussion

The aim of the present study was to investigate whether individuals with GS are protected against chromosomal damage. It was hypothesised that UCB, a concentration dependent antioxidant and moderately elevated in subjects with GS, lowers

the level of DNA damage. Demographic factors such as age, gender and BMI were also taken into consideration.

As expected and previously reported by many groups (23–25), the circulating UCB concentrations of subjects in the GS group were significantly higher as compared to the controls. Elevated bilirubin in these individuals is partly due to decreased enzyme activity of UGT1A1. As mentioned above, the impact of UCB concentrations on chromosomal damage has not been studied before, as per our knowledge. However, Grant and co-workers (17) analysed the relation between polymorphisms of UGT1A1 and MNi formation in lymphocytes (kinetochore positive and negative). They found no association between these parameters. This result is in agreement with our findings as we could not detect significant differences between the GS and the control group in regard to formation of MNi.

Very little information on the DNA protective properties of bilirubin is available. Bilirubin reduced the mutagenic effect of pro-oxidative tertiary-butyl hydroperoxide (t-BuOOH) in the *Salmonella*/microsome assay (9). In another Experiment, an increase of heme oxygenase 1 (HO1) was induced by hyperbaric oxygen (HBO) treatment. HO1 is the rate limiting enzyme in bilirubin production and no further increase of oxidative DNA damage was induced by HBO treatment with increased levels of this enzyme. These findings indicate that the increase of the UCB concentrations may account for the adaptive response (26).

Another interesting effect, which is caused by UCB *in vitro*, was the induction of tumour suppressor of p53 (27) and hyperphosphorylation of the retinoblastoma tumour suppressor protein (Rb) (28); both effects are causally related to the induction of apoptosis. It is also known that bilirubin induces apoptosis in cultured colon cancer cells (29). Our results indicate that the rates of MNi and NBuds are decreased in older GS individuals (≥ 30 years) in cells of epithelial origin and both endpoints were inversely correlated with age in the GS group. In the case of MNi, it is known that they are formed as a consequence of structural and chromosomal aberrations (14). Also, NBuds are associated with the alteration of DNA stability caused by amplified DNA (20). There is also evidence that these structures contain whole or fragments of chromosomes (30). In contrast to MNi, the relation of NBuds to human diseases is not known yet. In this context, it is interesting to note that epidemiological studies provide evidence that the risk of epithelial cancers, in particular lung and colon cancer, is decreased with increasing UCB levels and also partly in individuals with GS (4–7). Regarding the lower buccal MNC and NBud values in older GS subjects, we presume that the effect is caused by chronic (>10 years) exposure to mildly elevated UCB and due to the lower BMI observed in the GS group which, particularly at greater age, could contribute to prevention of cardiovascular diseases. High BMI is an independent risk factor, as obesity is well known to be correlated to chronic diseases (31, 32).

In regard to the association of UCB concentrations and induction of apoptosis, it is noteworthy that we found insignificantly higher rates of pycnotic cells in the mucosa of GS subjects. It has been postulated that pycnosis reflect apoptotic cells in epithelial tissue.

All results for CBMN and BMcyt, were within the normal range (12), and mean values in lymphocytes (MNi) and buccal cells (MNi and NBuds) were comparable with other Austrian studies (33, 34). The NDI was in the expected range for normal cell division and served as a quality control for the CBMN assay (12).

The effects of age and gender on chromosomal damage are well documented in the literature. It is known that MNi frequency in lymphocytes is increasing with age (14, 35), which was confirmed within this study. Furthermore, we found higher frequencies of NBuds (lymphocytes) in subjects older than 30 years. Multiple factors may explain these results with increasing age, such as accumulation of genotoxic agents, as well as an unhealthy lifestyle and micronutrient deficiencies (36, 37). It has been previously reported that higher MNi frequencies (PBL) are found among women (35, 36); this observation was also confirmed by our study. However, the effect of age for the endpoints of chromosomal damage (MN, NBuds and BNC) in buccal cells was significantly lower in all older individuals (≥ 30 years). No age effect in buccal MN and NBuds was observed for the control group, though a negative correlation was found in buccal MNC for the GS group, which might have influenced the age results in Table III. No age effect for buccal MN, NBuds and BNC was found by Rickes *et al.* 2010, although these authors investigated a broad age range of subjects—from 15 to 66 years (38). This was supported by the meta-analysis of Ceppi *et al.*, who also did not reveal a clear effect of age on buccal MNC (39). Buccal cells are known to have a lower repair capacity and a higher cell turnover rate (40), which might explain the different results of both cell types in the present study.

Table IV. UCB concentration, demographic features and nuclear anomalies in lymphocytes and buccal cells of older and younger GS individuals and corresponding controls

Parameters[a]		Controls <30 years (n = 21)	Controls ≥30 years (n = 17)	Gilbert's syndrome <30 years (n = 22)	Gilbert's syndrome ≥30 years (n = 16)
	UCB (µmol/l)	10.1 (3.21)	10.5 (3.52)	29.3 (12.3)[c,e]	35.6 (14.9)[c,e]
	Age (years)	24.1 (2.46)	41.5 (10.4)[b,c]	24.5 (2.60)	43.1 (10.9)[b,c]
	BMI (kg/m²)	23.0 (2.85)	26.1 (2.85)[b,c,d]	22.3 (2.84)	23.4 (2.83)
CBMN	MNC_PBL	6.40 (3.15)	12.6 (7.25)[b,c]	7.00 (3.13)	14.6 (7.94)[b,c]
	MNi_PBL	6.85 (3.36)	15.2 (10.6)[b,c]	7.45 (3.28)	17.1 (10.4)[b,c]
	NPBs_PBL	0.74 (0.45)	1.12 (0.78)	0.85 (0.88)	1.25 (1.65)
	NBuds_PBL	1.90 (1.29)	3.00 (1.84)	2.62 (2.06)	4.50 (3.50)
BMCyt	MNC_BC	0.23 (0.32)	0.22 (0.32)	0.45 (0.43)	0.12 (0.21)[b]
	MNi_BC	0.32 (0.48)	0.29 (0.41)	0.52 (0.48)	0.12 (0.21)[b]
	NBuds_BC	0.61 (0.59)	0.57 (0.69)	0.79 (0.48)	0.23 (0.29)[b]
	BNC	12.9 (4.16)	11.0 (4.25)	13.3 (5.12)	10.2 (5.09)

[a]CBMN values are per 1000 BN and BMCyt values are per 1000 differentiated cells; values as mean (standard deviation); LSD or Games Howell post hoc tests for differences from [b]GS <30 years, [c]controls <30 years, [d]GS ≥30 years, [e]controls ≥30 years; (p <0.05)

Taken together, the present findings show that nuclear anomalies which reflect DNA instability are decreased in older individuals with GS. This indicates that these subjects are protected against the consequence of variation in the genetic material; however, larger cross-sectional studies on the effects of age in subjects with GS are required.

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Original Article 2

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Cancer Prevention Research

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

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Research Article

Cancer Prevention Research

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 $\geq$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 (8oxodGuo, DNA oxidation) and 8-oxo-guanosine (8oxoGuo, 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 8oxodGuo concentrations were detected in the human group with a BMI $\geq$25 kg/m^2 (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

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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 (H_2O_2) 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 mm^2) 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

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carotenoids, 500 μL of the hexane phase was dried under nitrogen stream and dissolved in mobile phase (72% acetonitrile, 10% methanol, and 18% dichloromethane). The column (LiChrospher 100, RP-18, 5 μm, 250 × 4 mm^2, 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 mm^2; Merck, Hitachi, LaChrom). Potassium hydrogen phosphate buffer including 4% acetonitrile was used as mobile phase (24).

Animal study: design and treatment of animals

Twenty hyperbilirubinemic Gunn rats (homozygous for a mutation in *UGT1A1*, jj) and 20 respective controls (normobilirubinemic Wistar rats, heterozygous for a mutation in *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 ± 1°C, humidity 50% ± 20%, 12-hour light:dark cycle) and fed with a standard diet (ssniff R/M-H Extrudat, ssniff 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 rodents) or a treatment group (11 jj and 11 jJ rodents). The treatment group was γ-irradiated (^{60}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 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 SCGE 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 IXα UCB peak was 10.9 minutes. UCB acted as an external control and possessed an isomeric purity of >99% (for 3.3% IIIα, 92.8% IXα, and 3.9% XIIIα 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.

SCGE assay

The SCGE 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 H_2O_2 (100 μmol/L, all, *ex vivo*) and in rodents (22 rats of 40, *in-vivo*) by γ-irradiation (10.12 Gy). Thirty microliters (~1 × 10^6 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 (strand breaks, H_2O_2) or triplicates (FPG) were conducted. The H_2O_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–Smirnoff test. To determine differences between 2 groups, an independent

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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, H_2O_2, FPG-sensitive sites, and 8oxodGuo) and RNA (8oxoGuo) was found. Similarly, antioxidant and vitamin concentrations did not differ between the Gilbert syndrome and the control group (Table 1).

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

Parameters	Controls ($n = 38$)	Gilbert syndrome ($n = 38$)	*P*
Gender (male/female)	28/10	28/10	
Age, y	31.9 (11.2)	32.3 (11.8)	0.881
BMI, kg/m^2	24.4 (3.22)	22.8 (2.85)	**0.025**
UCB, μmol/L	10.3 (3.31)	32.0 (13.6)	**<0.001**
γ-GT, U/L	19.5 (7.08)	22.2 (14.4)	0.300
AST, U/L	24.3 (7.22)	26.7 (7.35)	0.165
ALT, U/L	21.7 (8.27)	25.5 (14.4)	0.166
LDH, U/L	157 (23.7)	163 (26.6)	0.315
ALP, U/L	64.0 (14.9)	70.1 (17.3)	0.105
Standard conditions[a,b]	5.08 (1.19)	5.13 (1.34)	0.862
H_2O_2-sensitive sites[a]	18.6 (4.49)	19.5 (4.80)	0.608
FPG-sensitive sites[a]	4.97 (3.07)	4.64 (2.58)	0.608
8oxodG[c]	1.86 (0.52)	1.86 (0.64)	0.466
8oxoGuo[c]	1.59 (0.54)	1.62 (0.70)	0.848
Vitamin C, μmol/L	73.2 (12.5)	71.5 (13.1)	0.570
β-carotene, μmol/L	0.59 (0.41)	0.61 (0.44)	0.794
Glutathione, mg/dL	74.2 (9.03)	72.0 (11.0)	0.329
Folic acid, ng/mL	142 (120)	142 (116)	0.608
Vitamin B12, ng/L	280 (125)	283 (121)	0.900

NOTE: Values as mean (SD).
[a]% DNA in tail.
[b]Strand breaks and apurinic sites.
[c]nmol/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/m^2, ≥25 kg/m^2) 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/m^2. 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

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Table 2. UCB concentration, demographic features, and DNA damage of controls and individuals with Gilbert syndrome subdivided in groups of age, gender, and BMI

	Age		Gender		BMI	
Parameters	**<30 y (*n* = 43)**	**≥30 y (*n* = 33)**	**Male (*n* = 56)**	**Female (*n* = 20)**	**<25 kg/m^2 (*n* = 53)**	**≥25 kg/m^2 (*n* = 23)**
UCB, μmol/L	19.9 (13.2)	22.7 (16.5)	22.4 (15.5)	17.5 (11.6)	23.6 (16.1)	15.3 (8.55)[h]
Age, y	24.3 (2.50)	42.2 (10.5)[d]	31.5 (11.4)	33.9 (11.7)	29.7 (9.86)	37.7 (13.0)[h]
BMI, kg/m^2	22.6 (2.83)	24.8 (3.11)[d]	23.8 (2.89)	22.9 (3.71)	21.9 (1.75)	27.4 (1.91)[h]
Standard conditions[a,b]	5.06 (1.31)	5.17 (1.27)	5.27 (1.21)	4.65 (1.31)[f]	4.93 (1.26)	5.51 (1.16)[i]
H_2O_2-sensitive sites[a]	19.6 (4.68)	18.3 (4.55)	19.1 (4.78)	19.0 (4.33)	19.5 (4.77)	18.1 (4.26)
FPG-sensitive sites[a]	5.13 (3.08)	4.38 (2.43)	4.98 (2.96)	4.31 (2.40)	4.71 (2.87)	5.02 (2.77)
8oxodG[c]	1.66 (0.60)	1.53 (0.66)	1.58 (0.65)	1.66 (0.56)	1.70 (0.67)	1.38 (0.43)[h]
8oxoGuo[c]	1.75 (0.49)	2.00 (0.66)[e]	1.78 (0.58)	2.10 (0.53)[g]	1.82 (0.55)	1.94 (0.64)

NOTE: Values as mean (SD).
[a]% DNA in tail.
[b]Strand breaks and apurinic sites.
[c]nmol/mmol creatinine.
[d]$P < 0.05$ from age < 30 years.
[e]$P = 0.067$ from age < 30 years.
[f]$P = 0.060$ from males.
[g]$P < 0.05$ from males.
[h]$P < 0.05$ from BMI < 25 kg/m^2.
[i]$P = 0.064$ from BMI < 25 kg/m^2.

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

	Wistar (*n* = 20)		Gunn (*n* = 20)		
Parameters[a]	**Without treatment (*n* = 9)**	**With treatment[f] (*n* = 11)**	**Without treatment (*n* = 9)**	**With treatment[f] (*n* = 11)**	***P* ANOVA**
Gender (male/female)	4/5	5/6	4/5	5/6	
UCB, μmol/L	0.58 (0.20)	0.55 (0.19)	104 (28)[c]	108 (26)[c]	<0.001
UCB min/max, μmol/L	0.40/0.92	0.23/0.85	71.3/167	73.3/169	
Body mass, g	206 (32)	209 (35)	186 (44)	200 (65)	0.699
PBMCs					
Standard conditions[b]	6.86 (1.16)[d]	20.5 (6.95)	9.20 (2.22)[d,e]	19.8 (6.88)	<0.001
FPG-sensitive sites	28.4 (6.32)[d]	54.5 (10.0)	33.6 (7.74)[d]	53.3 (6.99)	<0.001
Colonocytes					
Standard conditions[b]	16.9 (6.60)[d]	36.6 (5.22)	13.0 (2.88)[d]	36.8 (9.57)	<0.001
FPG-sensitive sites	18.4 (8.16)	20.4 (6.14)	20.0 (6.68)	20.8 (4.73)	0.508
Hepatocytes					
Standard conditions[b]	8.29 (1.41)[d]	28.1 (6.82)	8.76 (1.78)[d]	27.0 (1.78)	<0.001
FPG-sensitive sites	18.7 (6.17)	19.9 (4.74)	15.7 (3.41)[d]	22.7 (4.88)	<0.05

NOTE: Values as mean (SD).
[a]Unit of DNA damage parameters: % DNA in tail.
[b]Strand breaks and apurinic sites.
[c]Least significant difference (LSD) or Games-Howell *post hoc* tests for differences from Wistar groups.
[d]LSD or Games-Howell *post hoc* tests for differences from treatment groups.
[e]*t* test difference from Wistar without treatment.
[f]10.12 Gy radiation dose.

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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 8oxoGuo 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 *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 *UGT1A1*. This finding is in agreement with our results. A lower repair capacity and radiation sensitivity was seen in individuals with the *UGT1A1***28* polymorphism. Khan and Poduval (31) reported induction of strand breaks in human lymphocytes after incubation with 50 μmol/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 RNA 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 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

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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.

Authors' Contributions

Conception and design: D. Doberer, L. Vitek, A.C. Bulmer, K.-H. Wagner
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Original Article 3

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Original Paper

Protection from age-related increase in lipid biomarkers and inflammation contributes to cardiovascular protection in Gilbert's syndrome

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Abstract

Recent epidemiological and clinical data show protection from CVD (cardiovascular disease), all-cause mortality and cancer in subjects with GS (Gilbert's syndrome), which is characterized by a mildly elevated blood bilirubin concentration. The established antioxidant effect of bilirubin, however, contributes only in part to this protection. Therefore we investigated whether mildly elevated circulating UCB (unconjugated bilirubin) is associated with altered lipid metabolism. The study was performed on GS and age- and gender-matched healthy subjects ($n = 59$ per group). Full lipoprotein profile, TAG (triacylglycerols), Apo (apolipoprotein)-A1, Apo-B, lipoprotein(a), the subfractions of LDL (low-density lipoprotein) and selected pro-inflammatory mediators were analysed. A hyperbilirubinaemic rodent model (Gunn rats, $n = 40$) was investigated to further support the presented human data. GS subjects had significantly ($P < 0.05$) improved lipid profile with reduced total cholesterol, LDL-C (LDL-cholesterol), TAG, low- and pro-atherogenic LDL subfractions (LDL-1 + LDL-2), Apo-B, Apo-B/Apo-A1 ratio and lower IL-6 (interleukin 6) and SAA (serum amyloid A) concentrations ($P = 0.094$). When the control and GS groups were subdivided into younger and older cohorts, older GS subjects demonstrated reduced lipid variables (total cholesterol and LDL-C, TAG and LDL-C subfractions, Apo-B/Apo-A1 ratio; $P < 0.05$; Apo-B: $P < 0.1$) compared with controls. These data were supported by lipid analyses in the rodent model showing that Gunn rat serum had lower total cholesterol (2.29 ± 0.38 compared with 1.27 ± 0.72 mM; $P < 0.001$) and TAG (1.66 ± 0.67 compared with 0.99 ± 0.52 mM; $P < 0.001$) concentrations compared with controls. These findings indicate that the altered lipid profile and the reduced pro-inflammatory status in hyperbilirubinaemic subjects, particularly in the older individuals, probably contribute additionally to the commonly accepted beneficial antioxidant effects of bilirubin in humans.

Key words: apolipoprotein, cardiovascular disease (CVD), Gilbert's syndrome, low-density lipoprotein (LDL)-subfractions, lipid metabolism, unconjugated bilirubin.

INTRODUCTION

Strong epidemiological evidence indicates that circulating blood bilirubin protects from NCDs (non-communicable diseases) such as CVD (cardiovascular disease) [1] and cancer [2,3]. Furthermore, all-cause mortality in the general population [4] and in chronic haemodialysis patients [5] is negatively associated with plasma bilirubin levels. GS (Gilbert's syndrome) is a benign condition characterized by moderately elevated UCB (unconjugated bilirubin) levels ($\geqslant 17.1\ \mu$M) because of additional TA repeats in the gene promoter for bilirubin UGT (uridine diphosphate glucuronosyltransferase 1 family, polypeptide A1). Bilirubin conjugation and excretion into the bile is decreased thereby increasing the circulating UCB concentration. This polymorphism affects approximately 3–13% of the general population [6,7].

Thus far, a full explanation for the protective effects of bilirubin in large epidemiological studies is lacking and is mainly based on bilirubin's *in vitro* antioxidant capacity [8]. Investigations in

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; Apo, apolipoprotein; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; CVD, cardiovascular disease; γ-GT, γ-glutamyl transferase; GS, Gilbert's syndrome; HDL, high-density lipoprotein; HDL-C,HDL-cholesterol; IDL, intermediate-density lipoprotein; IL, interleukin; LDH, lactate dehydrogenase; LDL, low-density lipoprotein, LDL-C, LDL-cholesterol; NCD, non-communicable disease; SAA, serum amyloid A; TAG, triacylglycerol(s); UCB, unconjugated bilirubin; UGT, uridine diphosphate glucuronosyltransferase; UGT1A1, uridine diphosphate glucuronosyltransferase 1 family, polypeptide A1; VLDL, very-low-density lipoprotein.

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GS subjects, and experiments utilizing the rat model of hyperbilirubinaemia (Gunn rat), show protection from *ex vivo* [9,10] and *in vivo* [11] oxidation, coronary atherosclerosis [12], protection from thiol and protein oxidation [13] as well as anti-inflammatory [14] or anti-proliferative properties [15,16] of bilirubin.

However, evidence from an earlier meta-analysis indicates that circulating bilirubin alone could not account for all of the cardiovascular protection seen in hyperbilirubinaemic subjects [1]. These observations suggest other variables are interacting with serum bilirubin, resulting in cardiovascular protection. When exploring possible variables, lipid metabolism must be considered since it possesses great relevance to CVD protection. Very limited, but remarkable recent data support this link showing an impact of bilirubin on susceptibility of sd-LDL (small dense LDL) and circulating lipids to oxidation in individuals with GS [8,17]. Lower concentrations of the VLDL (very-low-density lipoprotein), IDL (intermediate-density lipoprotein) and LDL sub-fractions, in addition to reduced TAG (triacylglycerol) concentrations [18] and reduced total serum cholesterol [13] in GS subjects are reported. Particularly, the focused study of LDL subfractions is important because they can predict atherogenic risk of individuals better than LDL-C (LDL-cholesterol) [19]. Further evidence of the influence of bilirubin on lipid metabolism is shown in the Gunn rat model (equivalent to human Crigler-Najjar syndrome), where hepatic bilirubin glucuronidation is minimal resulting in high unconjugated hyperbilirubinaemia [10,20].

For other lipid-related biomarkers such as Apo (apolipoprotein)-A1, Apo-B, lipoprotein (a) and pro-inflammatory cytokines, reports in GS are lacking. The Apo-B/Apo-A1 ratio is an independent predictor of the vascular disease risk [21] and metabolic syndrome. IL-6 (interleukin 6), a pro-inflammatory cytokine, induces the hepatic synthesis of pro-inflammatory CRP (C-reactive protein), which is a known independent risk factor for CVD and Type 2 diabetes [22–23]. A novel pro-inflammatory lipolytic adipokine, expressed in human adipocytes is SAA (serum amyloid A). SAA is an acute-phase protein that is increased in obese subjects [24] and correlates with the presence of CVD [25].

Descriptive studies investigating GS, lack either a specific focus on lipid metabolism and inflammation and/or an appropriate number/matching of subjects. Therefore we investigated well-accepted biomarkers of lipid metabolism and adiposity and also novel lipid-associated CVD risk markers including 12 lipoprotein subfractions in a larger cohort of individuals with mildly increased UCB levels and compared them with age- and gender-matched controls. These human data are strongly supported by additional results from the hyperbilirubinaemic Gunn rat model.

MATERIALS AND METHODS

Subject recruitment

A total of 118 healthy subjects were recruited from the general Austrian population and met the inclusion criteria of normal liver function, absence of any underlying disease and an age range between 20 and 80 years. Reticulocytes, γ-GT (γ-glutamyl transferase), ALT (alanine transferase), AST (aspartate aminotransferase), LDH (lactate dehydrogenase), ALP (alkaline phosphatase), haemoglobin and haematocrit were measured at an initial screening examination. Subjects with liver, heart or kidney disease, haemolysis, diabetes, cholelithiasis, organ transplants, history of CVD, cancer, smoking (>1 cigarettes /day), alcohol consumption (>7 standard drinks/week), excessive physical activity (>10 h/week) and vitamin supplementation (4 weeks before the first blood sampling) or other prescribed medication (other than the contraceptive pill) were excluded. Allocation to the GS group was based on a fasting serum UCB concentration of $\geqslant 17.1$ μM measured by HPLC (see below). Subjects were age- and gender- (38 men and 21 women) matched in each group (total 118 subjects). Written informed consent was obtained from each patient, and the study was approved by the ethical committee of the Medical University of Vienna and the General Hospital of Vienna (#274/2010) and was performed according to the Declaration of Helsinki.

Sample preparation

An overnight fasting blood sample was collected into lithium heparin and serum vacutainers. Samples were stored on ice in the dark until further analysis. Blood collection tubes were centrifuged (15 min at 1350 ***g*** at 4 °C) and serum or plasma was divided into aliquots, used fresh or stored at -80 °C for further analysis. Erythrocytes from lithium heparin Vacutainers were washed three times with isotonic phosphate buffer, divided into aliquots and stored at -80 °C.

Animal experiments

Hyperbilirubinaemic Gunn rats [homozygous for a mutation in *UGT1A1* (uridine diphosphate glucuronosyltransferase 1 family), jj] and respective controls (normobilirubinaemic Gunn rats, heterozygous for a mutation in *UGT1A1*, jJ) from the same genetic background were obtained from Charles University in Prague (Prague, Czech Republic) and acclimatized in the breeding facility of the Medical University of Vienna (Himberg, Austria) 1 week prior to experiments. The animals were housed in plastic cages (Macrolon type IV; Techniplast), under standard conditions (24 ± 1 °C, humidity 50 ± 5 °C, 12 h light/dark cycle) and fed with a standard diet (ssniff R/M-H Extrudat; ssniff Spezialdiäten) and *ad libitum* access to fresh water. All experiments were carried out with 7–8-week-old rodents (18 males and 22 females; a total number of 40 rodents). The study was approved by the ethical committee of the Austrian Federal Ministry of Science and Research (BMF-66.006/0008-II/3b/2011).

Sample preparation

Animals were killed by decapitation. Blood was collected into sodium-heparinized tubes (Ebewe) and immediately transferred into ficoll containing tubes (Greiner Bio-One) for PBMC (peripheral blood mononuclear cell) isolation. Plasma was obtained after centrifugation, then divided into aliquots and stored at -80 °C until further analysis.

Blood biochemistry

Liver function enzymes (γ-GT, AST, ALT, LDH and ALP), Apo-A1, Apo-B and lipoprotein(a), serum TAG

(triacylglycerols), total cholesterol and HDL-C [HDL (high-density lipoprotein)-cholesterol] were analysed using routine diagnostic tests on Olympus 5400 clinical chemistry analysers (Beckman Coulter) and measured on the day of blood sampling. The quantitative analysis of lipoprotein families and the lipoprotein subfractions, VLDL, IDL1-3, LDL1, LDL2, LDL3-7, HDL the low-atherogenic lipoprotein profile (IDL3 and LDL1) and the pro-atherogenic lipoprotein profile (VLDL, IDL1-2 and LDL2-7) in serum from a subsample of 36 age- and gender-matched samples, was performed by the Lipoprint LDL system (Quantimetrix) [26]. In the same subsample, IL-6 (high-sensitive ELISA; eBioscience) and SAA (N Latex SAA®; DADE Behring) were determined.

Anthropometric measurements

All anthropometric measurements were obtained from participants who were lightly dressed without wearing shoes. Body height (m) was measured with a stadiometer (Seca, Modell 214) and body mass (kg) using standard analogue scales (Seca Selecta 791). BMI (body mass index) was calculated as kg/m^2.

Statistical analysis

All statistical analyses were conducted using SPSS (IBM statistics, Version 17.0). Normal distribution within the data set was tested by the Kolmogorov–Smirnoff test (KS). Therefore some variables were $\log_{10}$- (e.g. UCB) transformed and an inverse transformation ($1/x$) for age was conducted. To determine differences between two groups, an independent sample Student's t test (parametric data) or Mann–Whitney U test (non-parametric data) was conducted. Depending on the homogeneity of variance and normality of data within groups, one-way ANOVA (parametric data) or Kruskal–Wallis H test (non-parametric data) was used for multiple group comparisons. Student–Newman–Keuls post-hoc tests were performed after one-way ANOVA to determine group differences. Pearson coefficient (parametric data) or Spearman rho correlation (non-parametric data) tested the bivariate relationships between the independent and dependent variables. Forward stepwise regression analysis was used to predict the effect of multiple-independent variables on lipid variables. Data are expressed as means $\pm$ S.D., and a P value <0.05 was considered significant.

RESULTS

General subject characteristics

UCB concentrations were significantly higher in GS subjects ($P < 0.001$) as compared with controls. No differences in liver enzyme activities existed between the groups. After age and gender matching, GS subjects showed a trend towards lower BMI than the respective controls ($P = 0.075$).

Human and animal lipid data

Whole group comparisons indicated that GS subjects exhibited reduced total cholesterol, TAG and LDL-C concentrations compared with controls ($P < 0.05$; Table 1). LDL subfractions were significantly (LDL-1 and LDL-2; $P < 0.05$) or tended (LDL-3) to be lower in GS (Table 1). The total scores of pro-atherogenic markers (sum of VLDL, IDL-1, IDL-2 and LDL2–7) were not significantly lower in GS; however, the low-atherogenic parameters (IDL-3 + LDL-1) were significantly reduced in GS compared with controls ($P < 0.05$) (Table 1). Significantly reduced Apo-B and the Apo-B/Apo-A1 ratio ($P < 0.05$) (Table 1) in GS was also demonstrated.

Lipid status in Gunn rats and littermate controls strongly supported the different lipid biochemistry data in GS subjects. Hyperbilirubinaemic rodents had significantly reduced total cholesterol, TAG and HDL-C concentrations ($P < 0.05$). Hyperbilirubinaemia and hypocholesterolaemia in Gunn rats were associated with significantly reduced body mass in female rats only ($P < 0.001$; Table 2).

When analysing the entire cohort significant negative correlations between UCB and BMI ($r = -0.211$, $P < 0.05$), total cholesterol ($r = -0.245$, $P < 0.01$), LDL-C ($r = -0.243$, $P < 0.01$), TAG ($r = -0.248$, $P < 0.01$), LDL-1 ($r = -0.247$, $P < 0.05$), LDL-2 ($r = -0.272$, $P < 0.05$) and the low-atherogenic subfractions ($r = -0.276$, $P < 0.05$) were found. These data were corroborated by analyses of Gunn rat sera. In female Gunn rats, strong negative correlations between serum UCB and total cholesterol ($r = -0.882$, $P < 0.001$), TAG ($r = -0.502$, $P < 0.05$) and HDL-C ($r = -0.919$, $P < 0.001$) existed. In male Gunn rats, UCB was negatively correlated with TAG ($r = -0.649$, $P < 0.05$).

Forward stepwise regression analysis revealed that bilirubin explained 21% of the variance in LDL-C, 15% of total cholesterol, 4.5% of LDL-1 and 4% of LDL-C in GS subjects. However, bilirubin explained 44.6% of the variance in total cholesterol, 42.4% of HDL-C and 28.3% of TAG in rodents.

Since lipid parameters, as risk factors for CVD, rise with age, more specific analyses were conducted by dividing the human cohort into two age subgroups (age group 1: <30 years; age group 2: $\geqslant$30 years with a range from 30 to 72 years) as shown in Table 3.

For almost all lipid parameters the older controls showed either higher values than older GS subjects (i.e. significantly greater for total cholesterol, TAG, LDL-C, LDL-1, the low-atherogenic subfraction and the Apo-B/Apo-A1 ratio, $P < 0.05$; Table 3) or tended to be greater for VLDL-C and Apo-B. The older control group had significantly higher total cholesterol, TAG, LDL-C, LDL-subfractions (except LDL-3), Apo-B and the Apo-B/Apo-A1 ratio than the younger control group ($P < 0.05$). Interestingly, this age-related increase in these parameters was not observed between the younger and the older GS group. In order to determine whether GS individuals were protected from age-related dyslipidaemia, the age-associated increase in total cholesterol, LDL-C, TAG and HDL-C concentrations in addition to BMI were plotted against age in both groups (see Supplementary Figure S1 at http://www.clinsci.org/cs/125/cs1250257add.htm). These data indicate that significant age-dependent increases in these variables existed in both groups. However, the relationship between age and lipid variables remained offset, below the relationship in controls. Furthermore, despite remaining significant in both groups, the age-associated increase in BMI remained clearly lower in GS subjects ($r^2 = 0.29$, $P < 0.05$), compared with controls ($r^2 = 0.44$, $P < 0.01$).

Table 1 Demographic features, biochemical parameters, biomarker for fat metabolism and inflammation of individuals with GS and controls

Values are means (SD). *Significantly different from the GS group; †difference between GS and control group for trend ($P < 0.10$). Low-atherogenic score, IDL3 and LDL1; pro-atherogenic score, VLDL, IDL1–2 and LDL2–7.

Parameter	Controls ($n = 59$)	GS ($n = 59$)	*P* value
Age (years)	36.4 (13.6)	36.5 (13.4)	0.901
Sex (n) (male/female)	38/21	38/21	
BMI (kg/m^2)	24.4 (3.56)	23.2 (3.18)	0.075†
UCB (μM)	8.1 (4.14)*	28.4 (12.84)	<0.001
γ-GT (units/l)	20.7 (8.13)	27.9 (14.4)	0.246
AST (units/l)	25.2 (7.47)	28.0 (9.33)	0.083
ALT (units/l)	20.4 (9.33)	23.9 (13.7)	0.252
LDH (units/l)	174 (40.5)	178 (33.8)	0.321
ALP (units/l)	63.2 (15.4)	72.5 (28.7)	0.105
Total cholesterol (mM)	5.51 (1.04)*	5.10 (1.35)	<0.05
TAG (mM)	1.22 (0.56)*	1.02 (0.45)	<0.05
HDL (mM)	1.56 (0.37)	1.58 (0.37)	0.683
LDL (mM)	3.28 (0.86)*	2.93 (1.174)	<0.05
VLDL (mM)	0.68 (0.20)	0.62 (0.20)	0.168
LDL-1 (medium LDL) (mM)	1.11 (0.29)*	0.93 (0.26)	<0.05
LDL-2 (small LDL) (mM)	0.45 (0.25)*	0.33 (0.22)	<0.05
LDL-3 (very small LDL) (mM)	0.03 (0.04)	0.02 (0.03)	0.108
Pro-atherogenic score	85.1 (23.5)	75.5 (29.8)	0.202
Low-atherogenic score	58.0 (13.7)*	51.5 (13.5)	<0.05
Apo-A1 (mg/dl)	145 (20)	143 (18)	0.524
Apo-B (mg/dl)	92.4 (19.8)*	84.9 (23.7)	<0.05
Apo-B/Apo-A1 ratio	0.66 (0.16)*	0.61 (0.20)	<0.05
Lipoprotein (a) (mg/dl)	37.1 (34.7)	38.9 (46.1)	0.482
SAA (mg/l)	4.08 (1.79)	3.51 (0.79)	0.094†
CRP (mg/dl)	0.15 (0.17)	0.13 (0.16)	0.457
IL-6 (pg/ml)	1.19 (1.07)*	0.75 (0.39)	<0.05

Pro-inflammatory parameters

GS subjects also experienced significantly ($P < 0.05$) reduced concentrations of the pro-inflammatory cytokine IL-6 compared with controls. A similar trend was shown for SAA ($P = 0.094$; Table 1).

When considering age (threshold 30 years; Table 3) the older control group had the highest SAA, CRP and IL-6 concentrations when compared with the other age-subgroups (older/younger GS subjects and the younger control group; $P < 0.05$).

DISCUSSION

The results of the present study provide three lines of evidence to suggest protection from age-related dyslipidaemia/inflammation in GS. First, BMI, total cholesterol, TAG, LDL-C, LDL sub-fractions, Apo-B, the Apo-B/Apo-A1 ratio, SAA and IL-6 were significantly lower in aged GS compared with matched controls (Table 3). Secondly, the significant age-associated increase in total cholesterol, TAG, LDL-C, LDL sub-fractions, pro- and low-atherogenic lipid scores, Apo-B, SAA, IL-6 and CRP occurred in controls but not in GS (Table 3). Finally, the highly significant differences in lipid profile are presented in both male and female hyperbilirubinaemic Gunn rats (Table 2), providing a clear mechanistic link between bilirubin/UGT1A1 function and perturbation of lipid status. Together, these data provide the translational evidence that bilirubin or its metabolism modulates lipid metabolism in humans, which could contribute to reduced CVD risk in GS.

BMI

The GS and control groups in this study were matched for age and gender. Matched GS subjects tended to possess lower BMI, which can mainly be explained by the significant differences between the older subgroups ($\geqslant$30 years; Table 3 and Supplementary Figure S1). In general, the BMI was in the range of the Austrian population [27]. The older GS group did not differ significantly from the younger GS group in BMI; however, the older control group had a significantly higher BMI than the older GS subgroup and could be defined as being overweight (>25 kg/m^2), whereas the older GS subgroup was, by definition, normal (<25 kg/m^2). This observation is supported by other large epidemiological observations showing lower BMI in hyperbilirubinaemic subjects ([4,28]; see [30] for review). Interestingly, in Gunn rats, a difference in body mass was only found in female rats, which is supported by recent studies in a smaller group of aged female

Table 2 Body mass, UCB and lipid parameters in combined, male and female littermate-matched Gunn ($n = 20$) and Wistar ($n = 20$) rats

Values are means (S.D.).

(a) Combined

Parameter	Wistar ($n = 20$)	Gunn (n = 20)	*P* value
Body mass (g)	208 (33)	193 (55)	0.325
UCB (μM)	0.57 (0.19)	106 (26)	<0.001
Total cholesterol (mM)	2.29 (0.38)	1.27 (0.73)	<0.001
TAG (mM)	1.66 (0.67)	0.99 (0.52)	<0.001
HDL (mM)	1.28 (0.16)	0.60 (0.52)	<0.001
LDL (mM)	0.31 (0.22)	0.27 (0.27)	0.65

(b) Male rats

Parameter	Wistar ($n = 10$)	Gunn ($n = 10$)	*P* value
Body mass (g)	239 (18)	240 (48)	0.934
UCB (μM)	0.58 (0.18)	98 (20)	<0.001
Total cholesterol (mM)	2.45 (0.30)	1.98 (0.48)	<0.05
TAG (mM)	1.83 (0.54)	1.23 (0.51)	<0.05
HDL (mM)	1.32 (0.17)	1.13 (0.26)	0.73
LDL (mM)	0.41 (0.27)	0.38 (0.36)	0.86

(c) Female rats

Parameter	Wistar ($n = 10$)	Gunn ($n = 10$)	*P* value
Body mass (g)	182 (13)	155 (20)	<0.001
UCB (μM)	0.55 (0.20)	112 (30)	<0.001
Total cholesterol (mM)	2.17 (0.40)	0.69 (0.10)	<0.001
TAG (mM)	1.53 (0.74)	0.80 (0.45)	<0.001
HDL (mM)	1.26 (0.17)	0.17 (0.08)	<0.001
LDL (mM)	0.24 (0.14)	0.18 (0.12)	0.36

Gunn rats [13] and cross-sectional clinical studies [29] as recently reviewed [30]. A recent short-term weight loss study was effective in significantly increasing bilirubin levels back to the normal reference range. In this study, a linear relationship was observed between increase in total bilirubin concentrations and amount of weight loss [31].

These observations indicate that bilirubin is linked to weight or weight loss and that GS subjects have a lower BMI which, particularly at a greater age, could significantly contribute to prevention of CVD. A low BMI is an independent protector against NCDs, since overweight and obesity is correlated to higher mortality from NCDs [32].

Lipid metabolism

Focused reports on the relationship between elevated bilirubin, the metabolic state of GS and lipid data have not been published in the literature thus far, although scattered findings have been presented, particularly recently. For example, reduced circulating total cholesterol [17], LDL-C [18], TAG [33] and elevated HDL/LDL ratio [9] have been documented in GS, although findings are not always consistent between published studies [34], because of differences in the subject matching and cohort characteristics [30].

The results of the present study demonstrate that many pro-atherogenic risk markers of lipid metabolism are significantly reduced in GS subjects (Table 1). These observations were more pronounced in Gunn rats, which also showed significantly lower HDL-C (Table 2). An interesting observation was the specific effect in female Gunn rats, which were also lighter than littermate controls. This effect is comparable with a recently published study of homozygote animals reporting total cholesterol values one-third of heterozygote and wild-type animals [10]. These data could reflect a dose response of circulating bilirubin on lipid metabolism that is species independent. Mechanistically, altered lipid metabolism could reflect a role for either UGT1A1 or unconjugated bilirubin in modifying lipid metabolism and cholesterol excretion [30]. This observation probably explains the reduction in body mass in littermate-matched homozygote animals for which, until now no explanation has been found. The pronounced effect of the Gunn phenotype in female animals could suggest an additional role for oestrogen in enhancing the bilirubin or UGT1A1 effect. This could be mediated by competition of oestrogen and bilirubin for glucuronidation [35] by UGT1A1, which would also explain the slightly elevated bilirubin levels compared with male Gunn rats (Table 2). In humans there were no significantly stronger effects in women compared with men (results not shown).

The greater reduction in HDL (compared with LDL) in the Gunn rats is reflected by a greater contribution of HDL to total cholesterol in rats and suggests underlying perturbation of cholesterol metabolism/excretion/absorption within dysfunctional UGT1A1 syndromes (human and animals) as reported [11] and recently comprehensively reviewed [30]. One limitation is the higher UCB concentration in Gunn rats compared with GS; however, the general effect on lipid metabolism (hypocholesterolaemia) was similar to humans and other adult hyperbilirubinaemic animal models are not available. Evidence for the impact of bilirubin metabolism on lipoprotein assembly is described by an effect on LDL subfraction concentrations. Small dense LDL particles appear to confer a higher level of CVD risk than the larger less dense LDL particles and to be more susceptible to oxidative modification [36]. We used a new, recently published electrophoretic method, which enables the analysis of up to 12 lipoprotein subfractions [18]. Atherogenic lipoprotein profile is characterized by the presence of VLDL, IDL and the presence of small dense LDL lipoproteins. The sum of pro-atherogenic subfractions that consist of VLDL, IDL1–2 and LDL2–7 was not significantly lower in GS (Table 1). Closer evaluation of the data reveals a significant increase in pro-atherogenic subfraction values in older controls (18.4% higher than that of the young control group), which did not increase significantly in older GS subjects (compared with younger GS subjects; Table 3). Interestingly, this finding emphasizes the protection from age-related dyslipidaemia in GS. The concentrations of the low-atherogenic fractions in the young groups compared with the older GS group were similar; but, however, were significantly reduced compared with the older control group ($P < 0.05$; Table 3).

Further support of the protective effects of GS on age-related dyslipidaemia was revealed on correlational analysis. In control and GS subjects total cholesterol, LDL-C and TAG increased in an age-dependent manner; however, the relationship in GS subjects remained consistently offset below that of controls

Table 3 Effect of age on biomarkers of lipid metabolism and inflammation in GS and control subjects
Values are means (S.D.). [a]$P < 0.05$ compared with the control of the age group (young or old); [b]P for trend < 0.1 to control of the age group (young or old); [c]$P < 0.05$ to younger GS and control. *Pro-atherogenic score, VLDL, IDL1-2 and LDL2–7; †low-atherogenic score: IDL3 and LDL1. n.s., not significant.

Parameter	Controls <30 years ($n = 27$)	GS <30 years ($n = 25$)	Controls ⩾30 years ($n = 32$)	GS ⩾30 years ($n = 34$)	ANOVA P value
Age (years)	24.5 (2.4)	24.3 (2.4)	46.5 (10.6)[c]	45.3 (10.8)[c]	<0.001
BMI (kg/m^2)	22.6 (2.8)	22.1 (2.8)	26.0 (3.5)[c]	24.2 (3.2)[b,c]	<0.001
UCB (μM/l)	8.8 (4.2)	27.1 (12.0 [a]	7.5 (4.1)	29.5 (13.6)[a]	<0.001
Total cholesterol (mM)	4.97 (0.88)	4.54 (0.91)[b]	5.92 (0.97)[c]	5.30 (1.14)[a]	<0.001
TAG (mM)	1.10 (0.58)	0.95 (0.39)	1.31 (0.53)[c]	1.08 (0.50)[a]	<0.05
HDL (mM)	1.58 (0.35)	1.52 (0.27)	1.63 (0.44)	1.53 (0.38)	n.s.
LDL (mM)	2.76 (0.63)	2.46 (0.71)[b]	3.68 (0.80)[c]	3.08 (0.93)[a]	<0.001
VLDL (mM)	0.63 (0.21)	0.61 (0.18)	0.75 (0.15)	0.62 (0.25)[b]	0.122
LDL-1 (medium LDL) (mM)	1.03 (0.27)	0.93 (0.25)	1.21 (0.30)[c]	0.93 (0.27)[a]	<0.05
LDL-2 (small LDL) (mM)	0.4 (0.40)	0.28 (0.19)	0.51 (0.28)[c]	0.40 (0.24)	<0.05
LDL-3 (very small LDL) (mM)	0.04 (0.04)	0.01 (0.02)	0.03 (0.05)	0.03 (0.04)	n.s.
Pro-atherogenic score*	77.6 (23.1)	70.0 (23.2)	95.1 (20.7)[c]	82.6 (36.3)	<0.05
Low-atherogenic score†	53.9 (12.4)	51.5 (12.7)	63.4 (13.8)[c]	51.4 (14.8)[a]	<0.05
Apo-A1 (mg/dl)	141 (19)	139 (19)	150 (20)	145 (17)	n.s.
Apo-B (mg/dl)	83.6 (18.6)	79.4 (19.2)	98.8 (18.4)[c]	89.6 (26.4)[b]	<0.05
Apo-B/Apo-A1 ratio	0.60 (0.15)	0.58 (0.18)	0.69 (0.15)[c]	0.61 (0.21)[a]	<0.05
Lipoprotein (a) (mg/dl)	40.0 (38.6)	29.2 (28.2)	34.9 (31.9)	46.8 (55.9)	n.s.
SAA (mg/l)	3.47 (1.21)	3.42 (0.74)	4.75 (2.11)[c]	3.61 (0.87)[a]	<0.05
CRP (mg/dl)	0.14 (0.20)	0.12 (0.17)	0.16 (0.15)[c]	0.14 (0.14)	n.s.
IL-6 (pg/ml)	0.90 (0.71)	0.78 (0.43)	1.59 (1.36) [c]	0.72 (0.32) [a]	n.s.

(Figure S1). Indeed, when observing the absolute total cholesterol and LDL-C concentrations in aged GS and controls, aged controls were hypercholesterolaemic (⩾5.92 mM total cholesterol; ⩾3.68 LDL-C), as defined by the American Heart Association [37], however, age-matched GS subjects were not.

Generally, these results are in agreement with a recently published study, showing decreased small dense lipoprotein fractions in GS [18]. However, this study investigated only men and did not age match the subjects. Regardless, these data and those of Ocadlik et al. [18] support the impact of bilirubin metabolism on TAG and VLDL synthesis, potentially by down-regulating their synthesis/assembly.

Further biomarkers to describe the CVD risk include the structural proteins of lipoproteins. Apo-1 and -2 are the major structural proteins of HDL particles, whereas Apo-B is a major protein of every other lipoprotein particle but HDL. The Apo-B/Apo-A1 ratio is considered as one of the strongest plasma lipid-associated predictors of CVD risk [38], which indicates the balance between potentially atherogenic and anti-atherogenic particles [39]. GS subjects showed similar Apo-A1 and Apo-B concentrations to control subjects (Table 1). Similar to the above-mentioned observations, the older GS subjects were not significantly different to the younger cohorts in their Apo-B values, whereas the older control group showed significantly higher Apo-B than the younger subjects ($P < 0.05$). These data indicate protection from the age-related increase in plasma Apo-B concentration in GS. The Apo-B/Apo-A1 ratio was also lower in GS subjects ($P < 0.05$ compared with control group), and was shown to be highest in the older control group with a mean of 0.69 ± 0.15 compared with 0.61 ± 0.21 in the older GS group. This shows that the older control cohort was almost classified as being at medium risk of myocardial infarction, the criterion for which is determined by a ratio of 0.7 or higher [40], but the older GS subgroup was clearly at a lower risk level.

Pro-inflammatory markers

SAA is a pro-inflammatory adipokine in humans linked to obesity and is a predictor of CVD [24,41]. Adipose tissue is inflamed in obese individuals with increased secretion of pro-inflammatory and decreased expression of anti-inflammatory adipokines [24,25]. We report a trend towards reduced SAA levels in GS subjects (Table 1), which was significantly lower in the older GS compared with control subjects (Table 3). The same effect was evident for IL-6, with the older GS group having lower plasma levels than the older control group (Table 3). CRP showed a similar trend, but due to considerable variation, the results failed to show significance.

These data show that in older subjects that are traditionally assumed to possess a higher risk of CVD, GS individuals have lower levels of pro-inflammatory cytokines and do not experience the age-related induction of dyslipidaemia and inflammation. Elevated bilirubin has previously been associated with low CRP levels [42], which is a widely used diagnostic criterion to assess the inflammation status. However, this is the first study to report lower IL-6 concentrations in GS, which induces hepatic liberation of CRP into the circulation. Lower

levels of CRP and also IL-6 are related to a lower risk of CVD [22] and diabetes [23] and further emphasize the importance of these biomarkers in contributing to cardiovascular protection in individuals with mildly elevated unconjugated bilirubin levels.

Conclusions

The results of the present study show that elevated circulating bilirubin, particularly the benign condition of GS, is associated with reduced concentrations of lipid and inflammation biomarkers and a trend to decreased BMI. Furthermore, it is shown that older subjects, who are generally at greater disease risk, are likely to benefit more from a mild congenital hyperbilirubinaemia. These data are strongly supported by serum lipid analysis in Gunn rats, with more pronounced effects in the female animals. The observations of the present study could contribute significantly to revealing a new mechanistic explanation for the previously reported protective effects of hyperbilirubinaemia against NCDs in many published epidemiological studies. Such efforts could assist in developing novel strategies to mildly increase circulating bilirubin concentrations for protection against NCDs.

CLINICAL PERSPECTIVES

- Epidemiological evidence indicates that circulating blood bilirubin is strongly associated with protection from chronic disease; however, a mechanistic explanation for this protection remains unknown.
- The results in the present study reveal a novel link between bilirubin and lipid metabolism, which is clearly perturbed in individuals with GS and the hyperbilirubinaemic Gunn rat. A clear trend towards prevention of the age-related increase in lipid biomarkers and inflammation in GS individuals has revealed an important clinical observation in hyperbilirubinaemic individuals.
- This finding significantly contributes to a topic that has so far been neglected in clinical research and practice and suggests a new direction of discussing the disease prevention using a novel approach of studying persons with a benign condition that affects millions of people worldwide.

AUTHOR CONTRIBUTION

Marlies Wallner performed the human study, assisted in the animal study and performed statistical analysis; Rodrig Marculescu and Daniel Doberer wrote and submitted the human Ethics application, oversaw patient recruitment, assisted in the planning of the study and performed some of the lipid analysis; Michael Wolzt provided clinical staff and material for the clinical trial and assisted in subject recruitment together with Marlies Wallner and Karl-Heinz Wagner; Oswald Wagner provided clinical staff and material for the clinical trial; Libor Vitek provided the Gunn rats and proofread the paper before submission; Andrew Bulmer planned the studies together with Karl-Heinz Wagner, obtained the funding with Karl-Heinz Wagner, gave advice on the animal work, assisted with the statistical analyses and contributed to the paper; Karl-Heinz Wagner obtained the funding, planned the studies together with Andrew Cameron Bulmer, supported Marlies Wallner and wrote the paper.

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Original Paper

SUPPLEMENTARY ONLINE DATA

Protection from age-related increase in lipid biomarkers and inflammation contributes to cardiovascular protection in Gilbert's syndrome

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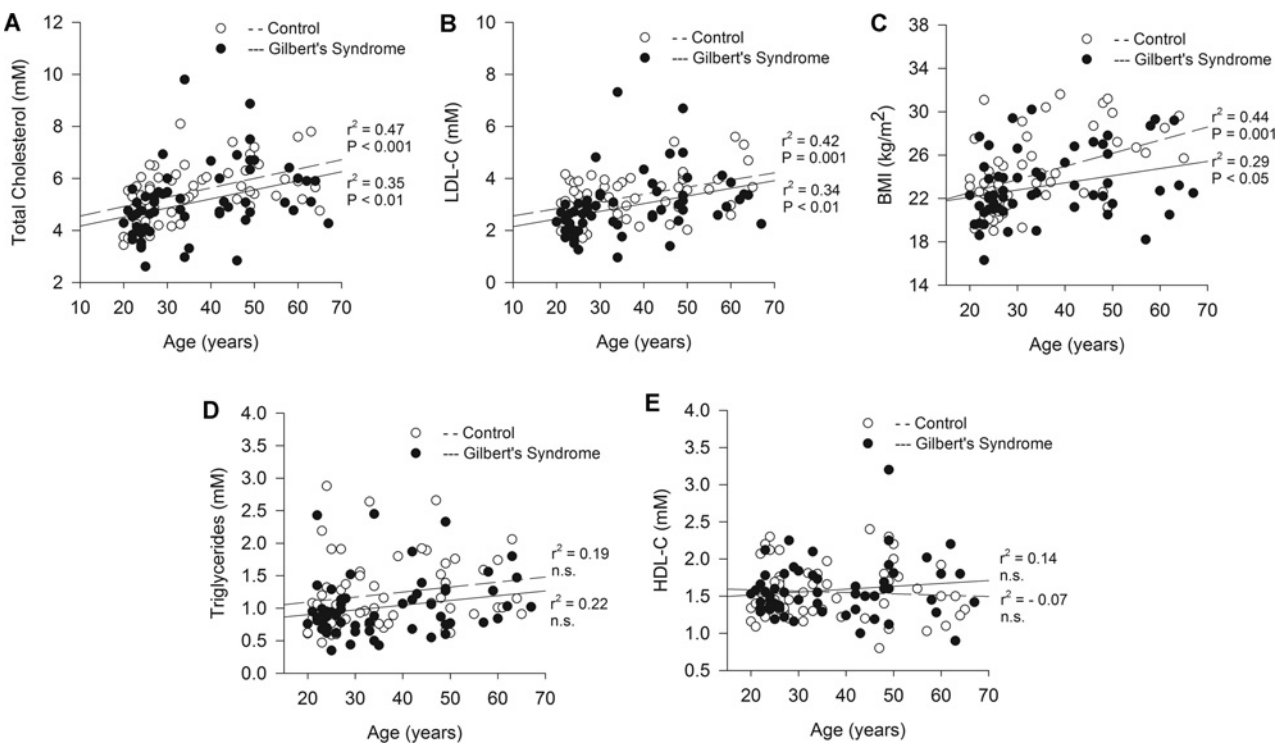

Figure S1 **Relationship between age and (A) circulating total cholesterol, (B) circulating LDL-C, (C) BMI, (D) circulating TAG and (E) circulating HDL-C in controls and matched GS subjects**

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4

Original Article 4

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ORIGINAL ARTICLE

Haem catabolism: a novel modulator of inflammation in Gilbert's syndrome

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ABSTRACT

Background Moderately elevated unconjugated bilirubin concentrations protect against inflammatory diseases and are present in individuals with Gilbert's syndrome. This study examined the relationship between circulating haem oxygenase catabolites, unconjugated bilirubin, carboxy haemoglobin, iron and inflammatory parameters.

Materials and methods Seventy-six matched individuals were allocated to Gilbert's syndrome (GS) or control group (unconjugated bilirubin $\geq$ or $<$ 17·1 μM). Iron, carboxy haemoglobin and high-sensitivity C-reactive protein were analysed using routine diagnostic tests. Unconjugated bilirubin and haem were analysed using high-performance liquid chromatography. The cytokines IL-1β, TNF-α and IL-6 were assessed using high-sensitivity enzyme-linked immunosorbent assays.

Results Gilbert's syndrome subjects had significantly greater levels of unconjugated bilirubin ($P < 0{\cdot}05$), carboxy haemoglobin ($P < 0{\cdot}05$), iron ($P < 0{\cdot}05$), IL-1β ($P < 0{\cdot}05$), a significantly lower body mass index ($P < 0{\cdot}05$) and IL-6 concentrations ($P < 0{\cdot}05$) vs. controls. Regression analysis revealed that unconjugated bilirubin mainly explained IL-1β results (16%), and body mass index+IL-6 predicted 26% of the variance in C-reactive protein concentrations.

Conclusions A positive relationship between unconjugated bilirubin and free plasma haem, iron and carboxy haemoglobin indicated a positive feedback loop of haem oxygenase induction possibly mediated by unconjugated bilirubin. Furthermore, reduced body mass index in Gilbert's syndrome individuals was linked to reduced inflammation status, which could be influenced by circulating haem oxygenase catabolites and contribute to reduced risk of noncommunicable diseases in this population.

Keywords Cytokines, Gilbert's syndrome, haem oxygenase, metabolic syndrome, obesity, unconjugated bilirubin.

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Introduction

Haem oxygenase (HO), the rate-limiting enzyme of haem degradation, is responsible for the generation of carbon monoxide (CO), iron and ultimately, unconjugated bilirubin (UCB) *in vivo*. Bilirubin is further conjugated by the enzyme UDP-glucuronosyl transferase (UGT) allowing its excretion from the body [1]. Moderately elevated bilirubin levels are found in subjects with Gilbert's syndrome (GS) which is associated with a polymorphism in the UGT1A1 gene promoter that leads to reduced enzyme function [2]. The major polymorphism (7/7 TA repeats) affects approximately 3–13 % of the general population (approximately 12·4% men, approximately 4·8% women) [3,4].

Circulating bilirubin is negatively correlated with the prevalence and mortality associated with inflammatory conditions such as CAD, atherosclerosis and myocardial infarction [5–7]. The mechanisms behind bilirubin's protection might include its potent antioxidant, anti-inflammatory and lipid-lowering effects *in vivo* [8–10]. In accordance with these observations, mildly elevated bilirubin levels are linked specifically to reduced risk of CVD and inflammatory bowel diseases in GS subjects [11,12].

[1]These authors Contributed equally to this work.

HO-1, the inducible isoform of HO, is up-regulated by a variety of factors including haem, oxidative stress [production of reactive oxygen species (ROS)], lipopolysaccharides (LPS), irradiation, drugs and cytokines in addition to its own catabolite, CO [1,13]. The induction of HO-1 leads to anti-inflammatory effects in rat lymphocytes [14] and also in liver transplanted rats that are mimicked, in part, by the administration of bilirubin [15]. Furthermore, anti-inflammatory properties of CO are reported throughout the literature [16–18]. A positive feedback loop of haem catabolite production on HO activity, as already indicated for CO, might be relevant for inducing HO's cytoprotective properties [19]. A positive feedback loop of bilirubin on HO induction has not yet been reported, however, might assist in explaining the prevention from chronic inflammatory diseases in GS.

C-reactive protein (CRP) is an independent risk factor for noncommunicable diseases (NCDs) [20] and is also negatively associated with circulating bilirubin concentrations [8,21]. Interleukin-6 (IL-6) is a potent inducer of CRP in the liver [22]. Therefore, we hypothesised that CRP and IL-6 concentrations would be lower in GS subjects.

So far, IL-6, IL-1β and tumour necrosis factor alpha (TNF-α) have not been reported in individuals with Gilbert's syndrome. Therefore, we investigated whether products of haem catabolism (haem, CO, iron, UCB) are associated with changes in inflammatory parameters (IL-6, CRP, IL-1β, TNF-α) in subjects with GS compared to age- and gender-matched controls.

Materials and methods

Study design-subjects

One hundred and four healthy subjects were recruited from the general Austrian population. Written informed consent was obtained from each subject. Seventy-six subjects met the inclusion criteria and possessed normal liver function, were aged between 20 and 80 years and were free from any underlying disease. Reticulocytes, γ-glutamyl transferase (γ-GT), alanine transferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), haemoglobin and haematocrit were measured during a screening examination. Subjects with liver, heart or kidney disease, haemolysis, diabetes, cholelithiasis, organ transplants, history of CVD, cancer, smoking (> 1 cig/day), alcohol consumption (> 7 standard drinks/week), excessive physical activity (> 10 h/week), any medication affecting liver metabolism and vitamin/antioxidant supplementation (4 weeks prior the first blood sampling) were excluded. Allocation to the GS group was based on a fasting serum bilirubin concentration of ≥ 17·1 μM measured by HPLC [23]. Subjects were age and gender (28 men, 10 women) matched in each group (total 76 subjects). This study was approved by the ethical committee of the Medical University of Vienna and the General Hospital of Vienna (# 274/2010) and performed according to the Declaration of Helsinki.

Sample preparation

A 24-h fasting (400 kcal food restriction) blood sample was collected into lithium heparin and serum vacutainers. Samples were stored on ice in the dark until further analysis. Blood collection tubes were centrifuged (15 min, 1006 *g*, 4 °C), and serum or plasma was aliquoted, used fresh or stored at −80 °C for further analysis.

Blood biochemistry

Liver enzymes γ-GT, AST, ALT, LDH, ALP and iron were analysed using routine diagnostic tests on Olympus 5400 clinical chemistry analyzers (Beckman Coulter) and measured on the day of blood sampling.

Unconjugated bilirubin and haem

Unconjugated bilirubin and haem were determined in serum immediately after centrifugation or within 1 week, if additional replicates were required. A high-performance liquid chromatograph (Merck, Hitachi, LaChrom, Vienna, Austria) equipped with a photodiode array detector (PDA, Shimadzu,) and a Fortis C18 HPLC column (4·6 × 150 mm, 3 μm) with a phenomenex C18 HPLC guard column (4·0 × 3·0 mm) was used (modified from Brower *et al.*[24]). An isocratic mobile phase perfused the column and contained 0·1 M n-dioctylamine in methanol/water (95 : 5; v/v) and glacial acetic acid (6·01 g/L). Unconjugated bilirubin was extracted from samples by mixing 40 μL serum with 160 μL mobile phase. After centrifugation, 50 μL of the supernatant was injected at a flow of 1 mL/min. Retention time (rt) of the IXα peak was 10·9 min. Unconjugated bilirubin acted as an external standard and possessed a purity of > 99% (3·3% IIIα, 92·8% IXα and 3·9% XIIIα isomers respectively; 450 nm; Frontier Scientific Europe, Carnforth, Lancashire, UK). Haem was determined at 400 nm, and haemin was used as an external standard (> 98% purity, rt 6·8 min., Frontier Scientific).

Carboxy haemoglobin

Carboxy haemoglobin (CO-Hb) was measured using a blood gas analyzer ABL 700 (Radiometer, Vienna, Austria) after collecting blood in heparinised syringes and measured immediately.

Cytokines (IL-1β, TNF-α, IL-6) and CRP

IL-1β and TNF-α were measured in heparinized plasma and IL-6 in serum using high-sensitive enzyme-linked immunoassays (ELISA, eBioscience, Vienna, Austria). All measurements were performed in duplicate. C-reactive protein was analysed using the high-sensitive CRP Latex immunoturbidimetric assay

on an Olympus 5400 clinical chemistry analyzer (Beckman Coulter, Vienna, Austria).

Statistical analysis

All statistical tests were completed using SPSS (IBM statistics, Version 17.0, SPSS, Chicago, IL, USA). Normal distribution within the data set was tested by the Kolmogorov–Smirnov test (KS) prior to analysis. If necessary, variables that were not normally distributed were $\log_{10}$ transformed. To determine differences between the GS and control group, independent samples *t*-tests (parametric data) or Mann–Whitney *U*-tests (nonparametric data) were conducted. Pearson coefficient (parametric data) or Spearman's correlation (nonparametric data) tested the bivariate relationships between variables. Forward stepwise regression analysis was used to predict the effect of multiple independent variables on dependent variables. Data are expressed as mean ± SD, and a *P*-value < 0·05 was considered to be statistically significant.

Results

General comparisons between the GS and the control groups are presented in Table 1. After age and gender matching, the BMI of the GS group was significantly lower compared to controls ($P < 0{\cdot}05$). Furthermore, GS subjects had higher iron ($P < 0{\cdot}05$), CO-Hb ($P < 0{\cdot}05$), IL-1β concentrations ($P < 0{\cdot}05$) and lower levels of IL-6 ($P < 0{\cdot}05$) compared to the control group (Table 1).

Correlations, within the whole cohort, between dependent and independent variables were then conducted (Table 2). Bilirubin was significantly and positively correlated with iron ($r = 0{\cdot}546$, $P < 0{\cdot}05$), CO-Hb ($r = 0{\cdot}313$, $P < 0{\cdot}05$), haem ($r = 0{\cdot}261$, $P < 0{\cdot}05$; Fig. 1a–c) and IL-1β ($r = 0{\cdot}312$, $P < 0{\cdot}05$). Furthermore, bilirubin was negatively correlated with BMI ($r = -0{\cdot}292$, $P < 0{\cdot}05$), IL-6 ($r = -0{\cdot}228$, n.s.) and CRP ($r = -0{\cdot}222$, n.s.; Table 2). In addition, relationships between IL-6 and iron ($r = -0{\cdot}240$, n.s.) and IL-6 and CRP ($r = 0{\cdot}372$, $P < 0{\cdot}05$) existed. CRP was positively associated with BMI ($r = 0{\cdot}429$, $P < 0{\cdot}05$; Table 2).

The relationship between bilirubin and inflammatory parameters within each of the control and GS groups was then investigated (Fig. 2a–d). This analysis revealed a significant negative relationship between bilirubin and IL-6 as concentrations of bilirubin increased to 17·1 μM in controls ($r = -0{\cdot}371$; $P < 0{\cdot}05$); however, a nonsignificant relationship was observed above 17·1 μM in GS subjects ($r = 0{\cdot}096$; $P > 0{\cdot}05$; Fig. 2a). Similarly, bilirubin was nonsignificantly negatively correlated with CRP in controls (−0·294; $P = 0{\cdot}086$) and in GS subjects ($r = -0{\cdot}109$, n.s.; Fig. 2b). IL-1β concentrations decreased significantly with increasing bilirubin concentrations in controls (−0·355; $P < 0{\cdot}05$) and then tended to increase with bilirubin

Table 1 Demographic features, biochemical and inflammatory parameters of controls and individuals with Gilbert's syndrome (GS)

Characteristic	Controls ($n = 38$)	Gilbert's syndrome ($n = 38$)	*P*-value
Gender (male/female)	28/10	28/10	
Age (years)	31·9 (11·2)	32·3 (11·8)	0·881
BMI (kg/m²)	24·4 (3·22)	22·8 (2·85)	**0·025****
Haem (μM)	0·51 (0·44)	0·64 (0·47)	0·242
Unconjugated bilirubin (μM)	10·3 (3·31)	32·0 (13·6)	**< 0·001**
Iron (μM)	22·4 (9·15)	32·3 (8·32)	**< 0·001**
CO-Hb (%)	0·91 (0·23)	1·06 (0·25)	**0·009*****
γ-GT (U/L)	19·5 (7·08)	22·2 (14·4)	0·300
AST (U/L)	24·3 (7·22)	26·7 (7·35)	0·165
ALT (U/L)	21·7 (8·27)	25·5 (14·4)	0·166
LDH (U/L)	157 (23·7)	163 (26·6)	0·315
ALP (U/L)	64·0 (14·9)	70·1 (17·3)	0·105
IL-1β (pg/mL)	2·07 (0·15)	2·21 (0·24)	**0·003*****
TNF-α (pg/mL)	2·65 (0·35)	2·54 (0·34)	0·171
IL-6 (pg/mL)	1·19 (1·07)	0·75 (0·39)	**0·027****
CRP (mg/dL)	0·16 (0·21)	0·09 (0·08)	0·413

The bold value denotes significant difference.
***P*-value < 0·05, ****P*-value < 0·01.
Data are presented as mean (SD).

Table 2 Bivariate relationships between independent and dependent variables

	Unconjugated bilirubin	TNF-α	IL-6	CRP
CO-Hb†	0·313***	–	–	–
Iron	0·546***	–	−0·240*	−0·209*
Haem	0·261**	–	–	–
BMI	−0·292**	–	–	0·429[c]
IL-1β	0·312***	0·213	0·242*	–
TNF-α	0·213	–	–	–
IL–6†	−0·228	–	–	–
CRP†	−0·222	–	0·372***	–

**P*-value < 0·1, ***P*-value < 0·05, ****P*-value < 0·01.
†variables transformed; if both variables were transformed or normally distributed, Pearson's correlation coefficient is presented; otherwise, Spearman's correlation coefficient was applied. Correlations only shown if $r > +/-0{\cdot}200$.

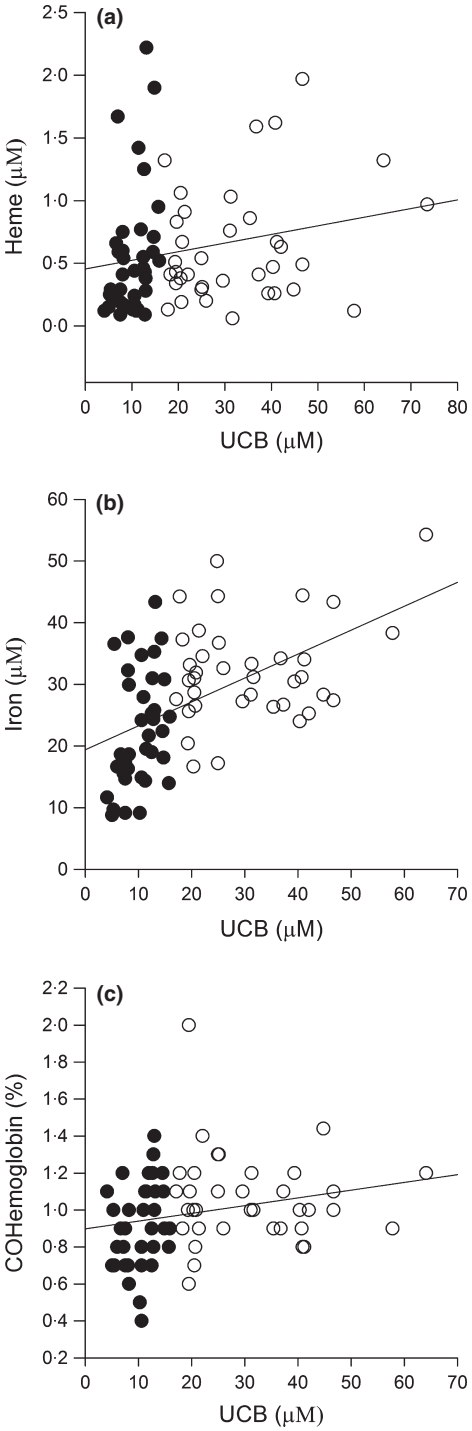

Figure 1 The relationship between circulating unconjugated bilirubin and haem ($r = 0{\cdot}261$; $P < 0{\cdot}05$; a), iron ($r = 0{\cdot}546$; $P < 0{\cdot}05$; b) and carboxy haemoglobin ($r = 0{\cdot}313$; $P < 0{\cdot}05$; c). Filled circles, control subjects; open circles, Gilbert's syndrome subjects.

concentrations in GS subjects ($r = 0{\cdot}315$; $P = 0{\cdot}062$; Fig. 2c). Finally, TNF-α concentrations nonsignificantly increased with bilirubin concentrations in controls ($r = 0{\cdot}134$; n.s.) and increased significantly with bilirubin concentrations in GS individuals ($r = 0{\cdot}344$; $P < 0{\cdot}05$; Fig. 2d).

Subsequently, multiple linear regression analysis was conducted to explore the ability of independent variables to explain inflammatory endpoints (Table 3). Only bilirubin (14·7%) could explain variations in IL-1β concentrations. Age estimated 18·8% of the TNF-α variance. The regression model for IL-6 included CRP and iron as independent variables and together predicted 27·1% of the outcome. The final model for predicting CRP included BMI and IL-6, which together explained 28% of the variance.

Discussion

This study demonstrates that elevated bilirubin is associated with increased blood CO-Hb, serum iron, haem, IL-1β and decreased IL-6 concentrations. Based upon these findings, we hypothesise that an association between bilirubin and CO-Hb/iron is due to a positive feedback loop of bilirubin on HO activation, mediated by a mild haemolytic effect of bilirubin (Fig. 3). We expect that this loop would be accentuated in GS, which independently increases circulating bilirubin concentrations [2], and therefore haem concentrations, and subsequently other HO catabolite concentrations (Table 1). Our results further show that the feedback loop exists in a continuum with low levels of CO-Hb and iron, progressively increasing with bilirubin load, evidenced by the positive correlation between bilirubin and CO-Hb/iron (Table 2). Furthermore, increasing CO-Hb concentrations might contribute to bilirubin production by inducing HO-1 [19].

We speculate that bilirubin might activate HO indirectly by increasing the fragility of red blood cells [25,26]. Therefore, further erythrocyte parameters were measured (Table S1) to confirm the absence of haemolytic disease in GS individuals. Despite otherwise normal haematology results, the serum haem concentration tended to be elevated in GS and was significantly positively correlated with circulating bilirubin concentrations in the entire cohort (Tables 1 and 2). This finding is corroborated by data from an additional clinical study, showing mildly elevated-free haem concentrations in GS individuals [23]. Therefore, we hypothesise that bilirubin induces a mild haemolytic effect, liberating haem, which is subsequently degraded to iron, CO and ultimately bilirubin. Carbon monoxide further induces HO-1 and accumulation of bilirubin, particularly in GS, inducing further red blood cell lysis, completing the loop of anti-inflammatory compound production.

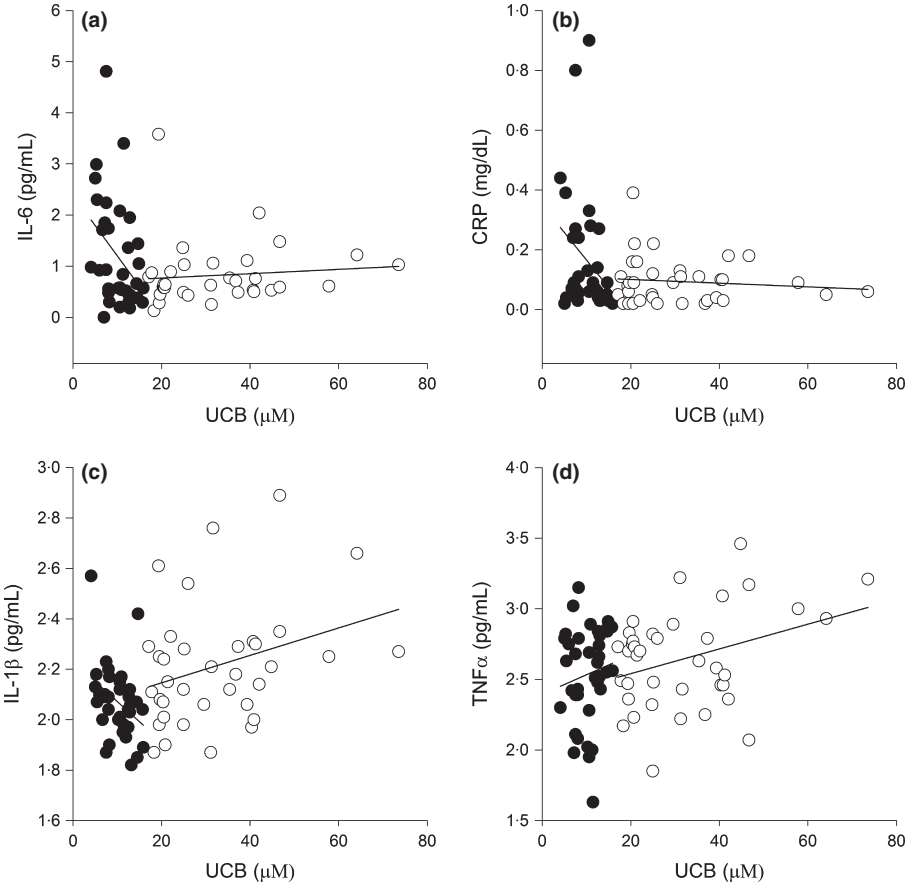

Figure 2 The relationship between circulating bilirubin and inflammatory biomarkers IL6 (a), CRP (b), IL-1β (c) and TNF-α (d) within control (filled circles) and Gilbert's syndrome subjects (open circles). R^2 and *P*-values for each regression are presented in the text of the results section.

Bilirubin and CO exert known immuno-modulatory effects [15–18]; therefore, elevated levels of these products combined could explain decreased IL-6 and increased IL-1β concentrations in GS. Our observation that IL-1β was positively correlated with bilirubin, in the whole cohort, was an unexpected finding in this study (Table 2). Bivariate analysis revealed that increasing bilirubin concentrations in controls (up to 17·1 μM) was associated with *decreased* IL-1β concentrations (Fig. 2). Within the GS group, however, as bilirubin concentrations *increased* above 17·1 μM, IL-1β concentrations tended to increase. This observation suggests that an optimal concentration of unconjugated bilirubin may exist *in vivo*, close to the cut-off for Gilbert's syndrome (17·1 μM). Bilirubin concentrations above and below this may be indicative of increased inflammatory status, revealing a 'U'- shaped relationship between bilirubin and IL-1β. Indeed, it has been proposed that a bilirubin concentration above 10 μM represents the value indicative of *decreased* risk for cardiovascular disease. However, these are the first *in vivo* data to suggest that bilirubin concentrations above 10 μM may be indicative of heighted inflammatory status. Interestingly, addition of unconjugated bilirubin to neonatal neutrophils increased IL-1β secretion and HO-1 gene expression, supporting our hypothesis of an important role for bilirubin in modulating haem catabolism and innate immune responses *in vivo* [27] (Fig. 3). The generation of mitochondrial ROS in macrophages is known to be induced by CO [28] and further leads to inflammasome activation with the release of IL-1β [29,30]. Indeed, bilirubin induces mitochondrial depolarisation in various cell types [31,32], providing a plausible mechanism linking elevated bilirubin to IL-1β release. Although IL-1β is thought to be a typical pro-inflammatory molecule, it might also prime neighbouring cells to increase their mitochondrial ROS production to resist pathogen invasion [29].

The present data are also supported by a clinical observation showing a relationship between the short repeat (GT)n polymorphism controlling HO-1 activity (leading to elevated circulating HO catabolites) and lower IL-6 concentrations in patients with coronary artery disease [33]. A recently published article linking this polymorphism to higher bilirubin levels [34], and a case report [35] supports an interaction between UGT1A1 and/or HO-1 with circulating bilirubin concentrations.

Unconjugated bilirubin is negatively associated with CRP levels [8], which is a widely used biomarker for inflammation

Table 3 Multiple regression analysis for cytokines as dependent variables

Dependent variables	Independent variables	R^2 (%)*	β†	*P*-value
IL-1β‡	UCB	14·5	0·006	0·001
TNF-α§	Age	18·8	0·014	< 0·001
IL-6¶	CRP	21·4	2·009	0·003
	+ iron	27·1	−0·026	0·026
CRP**	BMI	21·4	0·085	< 0·001
	+ IL-6	28·0	0·016	0·017

*R^2 adjusted correlation coefficient and cumulative explanation of dependent variable in %.
†Unstandardized B.
‡Excluded variables: age, sex, BMI, CO-Hb, iron, haem, TNF-α.
§Excluded variables: sex, BMI, UCB, CO-Hb, iron, haem, IL-1β.
¶Excluded variables: age, sex, BMI, UCB, CO-Hb, haem.
**Excluded variables: age, sex, UCB, CO-Hb, iron, haem.

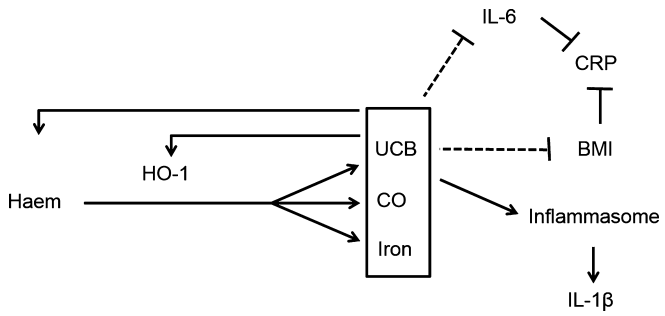

Figure 3 Hypothesised mechanism of bilirubin's involvement in a positive feedback loop on haem oxygenase (HO) activity, (i) Unconjugated bilirubin (UCB) induces a mild haemolytic effect, leading to haem degradation by HO and increased HO metabolite production (biliverdin/UCB, CO, iron). Bilirubin may also directly stimulate HO-1 transcription. (ii) Release of IL-1β. (iii) Elevated HO (end-) products contribute to a lower BMI and IL-6 concentrations as bilirubin concentrations increase and may act indirectly on C-reactive protein (CRP) production, by reducing IL-6 concentrations. Dashed lines indicate an effect specifically evident in Gilbert's syndrome.

status and CVD risk. Lower levels of CRP and also IL-6 are related to a reduced risk of suffering from NCDs [20,36]. Our results further support the observation that GS individuals have a significantly reduced risk of ischaemic heart disease [11], possibly due to lower IL-6 concentrations compared to controls. Importantly, we show that CRP levels were *not* correlated to bilirubin concentrations (either within the whole cohort or within individual groups), as reported previously [8]. However, BMI and IL-6 explained the variance in CRP results (Table 3). That elevated bilirubin is associated with reduced BMI in this and other studies [8,37], indicates that bilirubin is associated with lower CRP levels via reduction in blood lipid concentrations [10,23] and not by direct inhibition of inflammation. Interestingly, IL-6 is a known inducer of hepatic CRP production [38]; therefore, lower IL-6 levels in GS might provide a mechanistic explanation for reduced CRP concentrations in this cohort. Investigating the relationship between circulating bilirubin and IL-6/CRP indicated a stronger negative correlation within the control group compared to the GS group. These data indicate the importance of maintaining bilirubin concentrations above 17·1 μM, below which IL-6 concentrations increase, which might induce CRP synthesis. Furthermore, these data indicate that no additional benefit of increasing bilirubin concentrations above 17·1 μM exists on IL-6 concentrations, within the GS cohort.

The relationship between bilirubin and CRP appears stronger in females than males [8,37]. Recently, we have shown that body mass, serum cholesterol and triglyceride levels are more significantly decreased in female hyperbilirubinaemic Gunn rats vs. male animals [23,39]. These data further support a role for altered lipid status, induced by elevated bilirubin, in reducing CRP levels indirectly. This conclusion is further supported by a recent study that investigated the relationship between BMI, IL-6 and CRP plasma levels in obese and non-obese subjects [40]. A significant effect of BMI on CRP was reported, although no effect on IL-6 was noted. Therefore, the data presented here indicate that bilirubin could indirectly influence serum CRP by reducing BMI and IL-6.

In summary, these results are the first to suggest a potential effect of bilirubin on the production of anti-inflammatory haem catabolites via a positive feedback loop of HO induction (Fig. 2) *in vivo*, mediated by bilirubin-induced haemolysis. This effect could further amplify the anti-inflammatory and lipid-lowering effects of CO and bilirubin, particularly in GS. The novel finding that IL-6 and BMI were reduced in GS provides mechanistic insight into reduced CRP in this cohort. Finally, elevated IL-1β concentrations in GS and a positive relationship between bilirubin and IL-1β were an unexpected finding, the functional consequences of which require further investigation. Combined, these very novel observations strengthen the rationale for the pharmacologic manipulation of circulating bilirubin/biliverdin concentration [41] and provide further mechanistic insights into the reduced prevalence of NCD-related mortality in individuals with mildly elevated bilirubin concentrations.

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Author contribution

Marlies Wallner contributed to the study design, collected data, performed data analysis and interpretation and assisted in writing the manuscript; Andrew C. Bulmer received the grant with KHW, contributed to the initial study design, interpretation of data and data analysis and assisted in writing the manuscript; Christine Mölzer assisted in data analyses and reviewed/edited manuscript drafts; Elisabeth Müllner and Rodrig Marculescu collected data and reviewed/edited the manuscript drafts; Daniel Doberer contributed to the initial study design, was responsible for the lodgement of Ethics applications and oversaw subject recruitment; Michael Wolzt provided clinical staff and materials for the clinical trial and assisted in subject recruitment; Oswald F. Wagner provided clinical staff and materials for the clinical trial; Karl-Heinz Wagner directed the study, received the grant, contributed to the initial study design and reviewed/edited manuscript drafts.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Haematology parameters of Gilbert Syndrome and control subjects.