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„Evaluation of the DNA Repair capacity in subjects with Gilbert´s Syndrome "

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Petra Hinterleitner, BSc

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1 INTRODUCTION

The aim of the present Master´s thesis is to explore the protective role of bilirubin in the human organism. The human study was carried out at the Department of Nutritional Science, Vienna.

The endogenous bile pigment bilirubin is the degradation product of the heme catabolism and approximately 250mg per day is generated in adults. The enzyme UGT1A1 is responsible for glucuronidation of bilirubin in the liver and for excretion into the bile.

Gilbert´s syndrome (GS) is a hereditary and benign condition of moderate unconjugated hyperbilirubinaemia with a common prevalence in the population of up to 10%. The genetic background is based on a polymorphism located in the UGT1A1 gene, described as UGT1A1*28. Due to the polymorphism conjugation of bilirubin is reduced, which manifests in moderately elevated unconjugated serum bilirubin levels (≥17.1µM UCB) in GS subjects. In literature, GS is associated with a reduced risk for cardiovascular diseases (CVDs) and sometimes with a decreased prevalence of cancer.

The human organism is constantly exposed to exogenous and endogenous reactive oxygen species (ROS). DNA fragments, lipids and proteins are the cellular targets for oxidative attacks. Increased oxidative damage and decreased DNA repair capacity are considered as risk factors for the development of chronic diseases and for involvement in carcinogenicity.

Bilirubin has a strong antioxidant potential *in vitro* as well as in the human organism and could thereby reduce oxidative damage to macromolecules which in turn prevent oxidatively-induced chronic diseases. Additionally, bilirubin´s anti-mutagenic properties might also contribute to its preventive role in the development of chronic diseases, such as cancer. In this work, the hypothesis that GS subjects have a higher or more efficient capability to repair oxidatively-damaged DNA is tested. A higher DNA repair capacity in GS subjects might contribute to the clarification of bilirubin´s protective effect in the prevalence of

chronic diseases. Therefore, we allocated a study population, which consisted of 121 participants, based on their UCB concentration into gender- and age-matched groups, into the GS group (≥17.1µM UCB, n=61) and into the control group (<17.1µM UCB, n=60). Their DNA repair capacity was assessed with the *in vitro* Comet repair assay.

2 LITERATUR REVIEW

2.1 Oxidation Processes

The condition oxidative stress was first defined by Sies as “a disturbance in the prooxidant to antioxidant balance in favor of the oxidant species, leading to potential damage” (Sies 1991).

More recently, the term oxidative stress is also regarded as the disability of the organism to repair oxidative damage to biologically essential molecules (Kand'ar 2015). The cell´s anti-oxidative networks have the ability to scavenge excessively produced ROS. In some cases the balance is shifted in favor of free radicals, which over time leads to accumulated cell damage (Poljsak, Suput et al. 2013). Under normal physiological conditions the homeostasis among prooxidant and antioxidant substances favors prooxidant products (Droge 2002), as cells usually can handle mild oxidative stress levels. Such moderately increased levels are able to upregulate cellular repair processes and other protective systems (Poljsak, Suput et al. 2013).

Macromolecules, including DNA fragments, lipids and proteins are the cellular targets for oxidative attacks (Evans, Dizdaroglu et al. 2004). Depending on the degree of damage and the respective cell type, the consequences of oxidative stress are versatile and reach from increased genetic instability, proliferation, reduction of antioxidants, angiogenesis, to cell death and apoptosis and further play a role in carcinogenesis (Dizdaroglu 2015). Lenaz pointed out that oxidative stress is among the main reasons of toxicity to cells (Lenaz 2012). Oxidative stress is linked to more than 100 diseases, either as source or outcome (Pisoschi and Pop 2015).

Free radicals represent reactive oxygen species which possess an unpaired electron in the external orbit and therefore are able to exist independently (Pisoschi and Pop 2015). The presence of these unpaired electrons is responsible for the reactivity of free radicals (Valko, Izakovic et al. 2004). Reactive oxygen species include both free radical and non-free radical oxygenated molecules for instance hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), singlet oxygen (1/2 O_2), and the hydroxyl radical ($^{\bullet}OH$). In addition, there are

reactive nitrogen, iron, copper and sulfur species which are able to increase the level of ROS and oxidative stress and therefore are able to impair the redox balance (Poljsak, Suput et al. 2013).

Superoxide anions ($O_2^{\bullet-}$) are created either by metabolic processes or as a consequence of oxygen activation by irradiation. Superoxide anions are regarded as the “primary” ROS which can interact with other molecules to generate secondary ROS. This is commonly done by enzyme- or metal-catalyzed processes but can also be achieved in a direct way (Fridovich 1986).

The hydroxyl radical ($^{\bullet}OH$) is a highly reactive radical and accounts for oxidative damage to most biomolecules. This potent damaging radical arises from Fenton-type reactions and may also come from radiolysis of water (Gutteridge 1994). It has been pointed out that the $^{\bullet}OH$, at the source of its origin, interacts as the most powerful oxidizing radical with nearly all organic and inorganic molecules such as DNA, proteins, lipids, amino acids, sugars and metals. These processes are characterized by their fast reaction kinetics, the short life-span of hydroxyl radicals and by the inclusion of hydrogen abstraction, addition and electron transfer (Kohen and Nyska 2002).

Despite the fact that lifestyle plays an important role in health promotion we cannot avoid endogenous and exogenous free radical formation due to normal metabolism and exposure to environmental oxidants. The living cell is continuously exposed to potentially harmful free radical species originating from intracellular sources including the normal cellular metabolism and extracellular sources (Evans, Dizdaroglu et al. 2004). In general, free radicals are produced when cells oxidize nutrients, thereby producing energy. Mitochondria are identified as the most significant endogenous sources of oxidizing species which contribute to ageing (Gilca, Stoian et al. 2007). In addition, also many enzymes are capable of producing ROS including NADPH oxidase, xanthine oxidase, the alpha-ketoglutarat dehydrogenase complex, d-amino acid oxidase as well as dihydrolipoamide dehydrogenase (Gupta, Patel et al. 2014).

The effects of non-enzymatic antioxidants and antioxidant enzymes balance the impact of reactive oxygen and nitrogen species on cellular level. Obviously, this antioxidant defense is of great importance as it directly eliminates prooxidants, thereby providing maximal protection for biomolecules. The cellular redox homeostasis is maintained by a complex endogenous anti-oxidative defense system. Endogenous antioxidant enzymes like the superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione (GSH) and proteins belong to this system. In addition low molecular weight scavengers, for instance uric acid, bilirubin, coenzyme Q and lipoic acid, are also part of the antioxidant defense system (Halliwell 2011).

2.1.1 Oxidative DNA damage

Predicated on its molecular characteristics, DNA is highly prone to attacks of ROS (Choudhari, Chaudhary et al. 2014). Oxidative biochemical reactions result in the formation of reactive toxic intermediates which may result in DNA damage (Gupta, Patel et al. 2014) which play a crucial role in the pathogenesis of age dependent diseases such as arteriosclerosis, arthritis, neurodegenerative disorders and other conditions (Valko, Rhodes et al. 2006). It is expected that one human cell is exposed to nearly $1.5x10^5$ oxidative hits per day, resulting mainly from $^{\bullet}OH$ and other reactive species (Beckman and Ames 1997). Permanent alteration of genetic material due to oxidative damage represents the first step in mutagenesis, carcinogenesis and ageing. Increased levels of oxidatively modified DNA lesions are shown in many tumors. Further supportive findings suggest that oxidative mechanisms are often responsible for spontaneous mutagenesis. DNA damage due to ROS involves single- or double-strand breaks, purine, pyrimidine or deosyribose modifications and DNA cross links. Additionally, DNA damage can lead to transcription stop, the induction of altered signal transduction pathways, to replication errors and genomic instability, all of that are associated with carcinogenesis (Cooke, Evans et al. 2003). Indeed, it is not surprising that oxidative DNA damage is seen as a valuable indicator of oxidative stress and as a potential indicator of cancer risk.

Moreover, it has been shown that oxidative bases are elevated in diabetic and Alzheimer´s disease patients (Collins 2009).

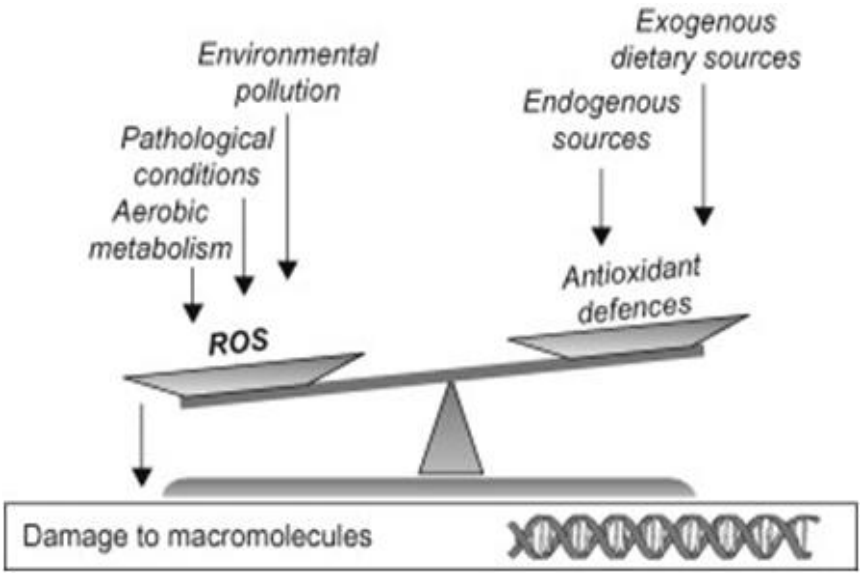

Figure 1. Imbalance between ROS and antioxidant (Iriti and Faoro 2010).

2.1.2 Oxidized purines, 8-oxodG

With regard to oxidative DNA damage the major scientific interest has been focused on oxidatively modified DNA bases (Valko, Izakovic et al. 2004). The purine base guanine has the lowest oxidation potential and is therefore most prone to oxidation (Klungland and Bjelland 2007). More than 20 base lesions are identified whereas 8-oxo-2´deosyguanosine (8-oxodG) is the most notable one (Cooke, Evans et al. 2003). For instance, in individuals exposed to tobacco smoke, the formation of 8-oxodG is increased by 35–50% (Sies 1997).

The $^{\bullet}$OH molecule interacts with all components of the DNA and is therefore able to damage both pyrimidine and purine bases as well as the deoxyribose backbone. As a consequence the addition of $^{\bullet}$OH to double bonds of DNA bases leads subsequently to the abstraction of the H atom from the methyl group of thymine and from each of the five carbon atoms of 2´-deoxyribose (Dizdaroglu, Jaruga et al. 2002). By binding the H atom (from the $^{\bullet}$OH molecule)

to the DNA base guanine C4-OH-, C5-OH- and C8-OH- adduct radicals are formed. The majority of guanine products in DNA arise from the reactions of the C8-OH-adduct radical. This one-electron oxidation gives rise to 8-oxodG (Dizdaroglu 2015).

Finally, 8-oxodG is easily generated and may reflect mutagenic and carcinogenic processes. Hence, 8-oxodG acts as eligible biomarker for oxidative stress of an organism and further as a potential biomarker reflecting carcinogenesis (Valko, Rhodes et al. 2006).

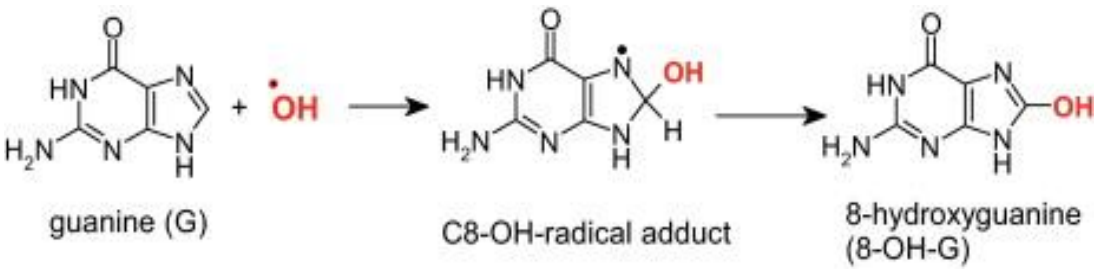

Figure 2. Reaction of guanine with hydroxyl radical (Valko, Rhodes et al. 2006).

2.1.3 Determining DNA damage using a whole cell approach

Ostling and Johanson were the first to develop an assay for quantifying DNA damage in cells by using a microgel electrophoresis technique, referred to as single cell gel electrophoresis (SCGE) or alternative Comet assay (Ostling and Johanson 1984). First, the technique was performed under pH-neutral conditions which allows only for detection of DNA double strand breaks (DSBs). A more sensitive version was adapted, in that alkaline conditions enabled the detection of both DNA double- and single-strand breaks (SSBs) (Dhawan, Bajpayee et al. 2009). With this international accepted method it is even possible to detect a variety of oxidized DNA lesions (Collins, Koppen et al. 2014). It is used in human biomonitoring studies, e.g. for occupational exposure to mutagens, for analyzing dietary interventions with antioxidants and for estimating levels of oxidative stress in relation to diseases. Furthermore, it is conducted in *in vitro* and animal models as it is simple, sensitive and rapid (Dusinska and Collins 2008).

The main principle of the assay is as follows. Human cells, peripheral blood mononuclear cells (PBMCs), are embedded in agarose and subsequently placed on a microscope slide, to yield small gels. After that cells are lysed using a lytic detergent and a high salt concentration (NaCL >2M) to remove the cell- and nuclear-membranes and to release all soluble cell components. DNA attached to the nuclear matrix as a nucleoid is left behind and supercoiling of the DNA is still maintained. When alkaline electrophoresis (pH value >13) is applied, those DNA loops that are damaged, lose their supercoiling and extend towards the anode by forming a visible “comet tail”. In brief, the more DNA breaks are available, the more supercoiled DNA loops are released and the more DNA appears in the comet tail (Collins, Dusinska et al. 1997).

2.1.4 DNA Repair Processes

Cellular signaling responses from cells, including damaged DNA, and the ability to repair such damage are fundamental processes and crucial for the organism´s viability (Jeppesen, Bohr et al. 2011). DNA repair is one of the key elements in the dynamic balance that is regarded as genetic stability. In healthy individuals, the level of DNA damage is more or less constant, meaning that the damage occurring due to environmental exposure or internal events is balanced by different repair mechanisms (Collins and Azqueta 2012). Oxidatively-damaged DNA must be repaired before replication and cell division takes place (Klungland and Bjelland 2007). The human enzyme glycosylase scans the DNA for damaged bases and initiates a repair pathway. Among these, the human 8-oxoguanine glycosylase (hOGG1) is the best studied one (Kubota, Nash et al. 1996).

It is of great importance to develop functional assays which can estimate the DNA repair capacity for identifying high-risk subgroups among the population (Berwick and Vineis 2000). Insufficient DNA repair mechanisms can lead to decreased stability of the cellular chromosomes, to cellular dysfunction, and to different phenotypes (Jeppesen, Bohr et al. 2011). For example, the autosomal recessive disorder Xeroderma pigmentosum (XP) makes individuals highly susceptible to skin cancer (2000-fold higher risk) due to mutations in the genes which are involved in the repair pathway nucleotide excision repair (NER) (Nagel, Chaim et al. 2014).

2.1.5 DNA Repair Pathways

Cells contain repair systems which recover nearly the whole damage before changes in the genome can occur. The various repair pathways include many groups of repair enzymes which are able to deal with different types of DNA modifications:

- single-strand breaks in the sugar-phosphate backbone are managed by insertion of one or a few bases and subsequent ligation;
- more serious DSBs are repaired by homologous recombination (HR) and non-homologous end-joining (NHEJ) DNA repair pathways;
- base excision repair (BER) deals with small base modification like alkylation or oxidation and SSBs;
- the most complex repair pathway NER handle bulky adducts of different molecules;
- mismatch repair (MMR) deals with incorrectly paired bases (Azqueta, Slyskova et al. 2014).

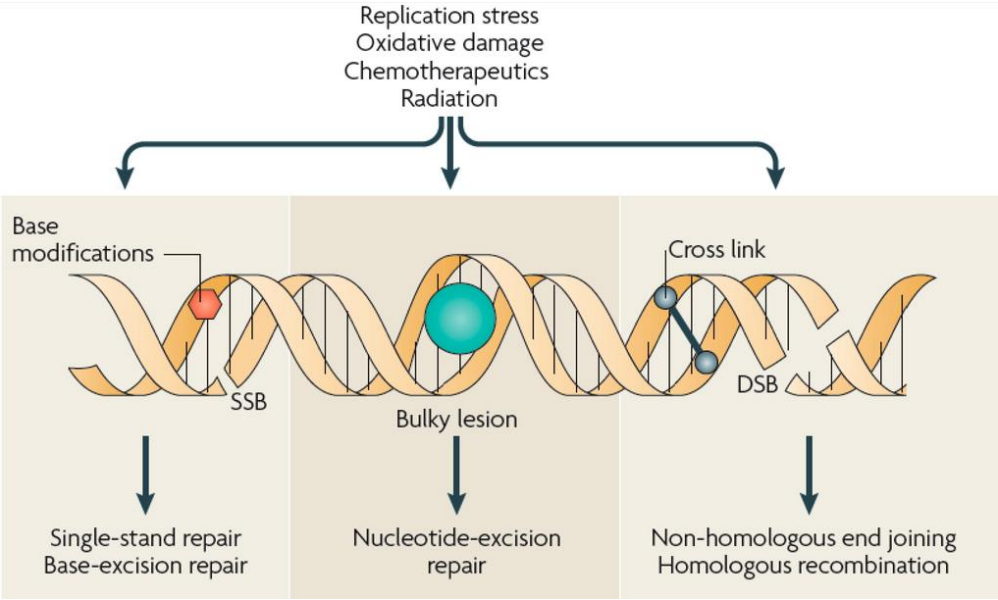

Figure 3. Types of DNA damage and repair (Arjunan, Sharma et al. 2015).

2.1.6 Base excision repair (BER)

The repair pathway BER plays a crucial part in preventing mutations due to its capability to repair modified bases, like 8-oxoG (David, O'Shea et al. 2007). The majority of small base modifications and nearly all oxidatively induced DNA lesions are repaired by the BER pathway (Altieri, Grillo et al. 2008), which is also needed for a normal mammalian development. Defective BER has been linked to neurological disorders and cancer (Wallace, Murphy et al. 2012).

The BER pathway primarily manages non-bulky small nucleobase lesions, excises and replaces incorrect or damaged bases which arise from deamination, alkylation or oxidation (Kim and Wilson 2012). Furthermore, BER is involved in the repair of SSBs (Jeppesen, Bohr et al. 2011).

The repair process of BER can be divided into two main parts. The first step includes the recognition and the excision of the damaged nucleobases completed by a DNA-glycosylase enzyme. The second step involves the replacement of the resulting “hole” with a correct nucleobase and is achieved by a variety of enzymes. DNA glycosylases, such as hOGG1, are the initiating enzymes in the BER pathway. They specifically recognize and remove modified nucleobases and form an abasic site (apurinic/apyrimidic, AP site). A distinguishable feature of DNA-glycosylase is that they are either monofunctional (just the DNA lesion is removed) or bifunctional (posses additional AP-lyase activity which is capable of converting the base lesion into a SSBs). The enzymes of the second step – AP-endonuclease, DNA-polymerase and DNA-ligase – process the AP-sites and repair the DNA structure. The BER mechanism includes a short-patch and long-patch pathway. The short-patch pathway can be introduced by the bifunctional glycosylase whereas the monofunctional glycosylase is able to initiate both pathways (Dizdaroglu 2015). Nearly all DNA-glycosylases which take part in the repair of oxidatively induced DNA damage possess AP-lyase activity and are bifunctional. The hOGG1 is mainly responsible for the repair of oxidized guanines, like 8-oxoG (Krokan, Standal et al. 1997).

The enzyme ß-polymerase (ß-pol) is deeply involved in the BER pathway. An animal study demonstrated that increased levels of 8-oxoG are associated with upregulated levels of ß-pol and BER capacity. It can therefore be concluded that ß-pol and BER may be upregulated as a consequence of oxidative stress *in vivo* (Cabelof, Raffoul et al. 2002).

2.1.7 Measuring DNA repair capacity at a cellular level (*in vitro* Comet repair assay)

The standard version of the SCGE assay (Comet assay) has been further developed and modified in order to measure the repair capacity of cells. Cellular repair activity can be monitored *in vitro* by the removal of specific DNA lesions that had been introduced. Compared to the standard comet assay one additional step is required: lesion-containing nucleoids are digested with lesion-specific endonucleases to detect different DNA lesions. The bacterial counterpart to hOGG1 is formamidopyrimidine DNA glycosylase (FPG) which detects formamidopyrimidines (i.e. ring-opened purines derived from oxidized adenine und guanine). The *in vitro* comet repair assay is evaluated primarily to study BER of 8-oxoG (Collins 2014).

The key principle of this biochemical approach is to collect a cell substrate that contains a known level of DNA lesions. This cell substrate is embedded in agarose and incubated with a cell extract of interest (human PBMCs samples) which contains unknown amounts of repair enzymes. Lysis of the lesion-containing substrate provides nucleoids. During incubation with the defined cellular extract, the initial step of BER takes place: the incision of the DNA at the place of lesions and the induction of SSBs. Subsequently, the standard alkaline electrophoresis determines the accumulated SSBs which were introduced by the repair enzymes (repair incisions). The repair pathway of interest depends on the type of lesion induced in the substrate: the BER pathway can be measured by 8-oxoG lesions which are induced by the photosensitiser Ro 19-8022 and light. The NER pathway can be evaluated by measuring nucleoids which contain dimerized pyrimidines caused by ultraviolet light (UV) (Azqueta, Slyskova et al. 2014).

The accumulated breaks form a “comet tail”. For analysis cells are stained with a suitable DNA-binding dye (e.g. gel red) and comets are analyzed with a computer-based fluorescence microscope. The relative intensity of fluorescence in the comet tail represents the DNA break frequency and is expressed as % DNA in tail (Azqueta and Collins 2013). Increased % DNA in tail is

corresponding to repair incision activity of the cell extract (Azqueta, Langie et al. 2013).

Indeed, using the comet repair assay for repair studies has limitations as it is possible to provide an indirect estimation for the speed of SSBs rejoining but no information about the final repair accuracy (Decordier, Loock et al. 2010).

2.1.8 DNA repair capacity *in vivo*

The DNA repair capacity is measured in human bio-monitoring studies, mainly for the purpose of defining markers of cancer susceptibility. DNA damage is the initial step in carcinogenesis: the faster the damage is repaired the less mutations can occur (Collins and Azqueta 2012). Neurological disorders, immunological disorders and premature ageing are related to alterations in DNA repair capability. Mutations which occur in the repair genes can lead to severe diseases (Nagel, Chaim et al. 2014).

The exponentially increasing number of studies, cancer case-control and environmental exposed-control studies, reflect the importance of analyzing DNA repair capacities (Decordier, Loock et al. 2010).

Gaivao et al. performed a study evaluating BER and NER activity in human lymphocytes in healthy subjects using the comet repair assay. They observed a four-fold increase in inter-individual variations in the activity of BER to repair 8-oxoG lesions; variations in the NER pathway were even higher. It was concluded that the *in vitro* comet repair assay is an appropriate method for biomonitoring studies (Gaivao, Piasek et al. 2009). Measuring these inter-individual differences provide basic insights into individual disease susceptibility (Nagel, Chaim et al. 2014).

Two studies have been conducted to investigate the repair capacity of bleomycin-induced DNA damage. Bleomycin is seen as an appropriate agent for evaluating DNA damage and repair due to its radiomimetic properties and because it is commonly used for cancer treatment. Repair rates were observed in peripheral blood lymphocytes (PBL) of non-small cell lung cancer and breast

cancer subjects and compared to healthy controls. Both studies reported slightly reduced repair capacity of bleomycin-induced DNA damage in the cancer subjects compared with the healthy controls (Jałoszyński, Kujawski et al. 1997, Rajaee-Behbahani, Schmezer et al. 2001).

A study conducted by Humphreys et al. provides interesting results with regard to DNA repair ability and the ageing process. Using the comet repair assay, they examined the oxidative DNA damage, the anti-oxidative status, and the DNA repair activity in lymphocytes from 3 different age groups (20–35 years, n=40; 63–70 years, n=35; 75–82, n=22). The older subgroup had been part of the Boyd Orr cohort (BOC) study, completed in the year 1930, to evaluate diet and health in the UK. Oxidative base damage seemed to be increased in older subjects, however, was not based on a decline of antioxidant defense or inefficient DNA repair. Indeed, the oldest age group shows a greater level of antioxidant defense and the DNA repair activity increases with age in all age groups. One possible explanation for such an unexpected and remarkable result is provided by the authors. It seems plausible that, in older people, antioxidant and DNA repair mechanisms are upregulated to cope with the higher levels of oxidative damage. Higher levels of oxidative damage may be explained due to the higher release of ROS from leaking mitochondria (Humphreys, Martin et al. 2007).

Soares and colleagues underline these results with similar findings. Sixty one Causcasian men were divided into two age groups (<65 years, n=43; ≥65 years, n=18). The aim of the study was to evaluate the influence of lifestyle and age on DNA damage and repair capacity. The older male subjects had higher DNA strand breaks and DNA repair capacity (BER) than the younger group. Both factors are positively correlated with age. Results suggest that DNA SSBs increases during the process of ageing, but there is no increase in FPG-sensitive sites (Soares, Silva et al. 2015).

In a population-based study including 216 subjects, 68% women and 32% men aged between 40–77 years, it was suggested that the repair of SSBs is maintained throughout the whole ageing process. In contrast, it was noted, that

the ability to repair DSBs decreases during the process of ageing. Moreover, no gender differences between the analyzed repair parameters (SSB- and DSB-repair) could be observed (Garm, Moreno-Villanueva et al. 2013).

A study assessed the level of genomic damage in chronic renal failure patients by the use of the comet assay. Further they evaluated if differences in the level of DNA damage are due to differences in the base excision repair capacity. Initially, the level of DNA damage was analyzed in 106 hemodialysis patients. For evaluation of the DNA repair capacity they used a subgroup of 21 patients. They compared patients with high levels of DNA damage (n=10) with patients who showed low levels of DNA damage (n=11). They demonstrated that the repair capacity was significantly lower in patients with higher levels of DNA damage in comparison to the repair capacity of patients with lower levels of DNA damage (12.73±1.84 vs. 18.13±1.13). Moreover, they observed a negative correlation coefficient between the two parameters (r=-0.423; p=0.056) (Stoyanova, Pastor et al. 2014).

Szymczak et al. conducted a study which assessed the level of DNA damage and DNA repair capacity in lymphocytes of patients with type 2 diabetes, with colorectal cancer (CRC), and in lymphocytes of healthy subjects (total of 32 subjects). For analyses, they used the comet assay. They indicated that patients suffering from type 2 diabetes and from coexisting CRC showed the highest level of DNA damage. The DNA repair capacity was significantly decreased in CRC patients with and without type 2 diabetes (Szymczak, Sliwinska et al. 2014).

2.2 The bile pigment Bilirubin

Bilirubin is a linear tetrapyrrole and the end product of heme catabolism. Heme is released after the breakdown of red blood cells by phagocytic cells (Jansen and Bittar 2004). In adults approximately 250mg/day of bilirubin is generated, whereas about 75% comes from circulating hemoglobin. The remaining 25% is derived from the catabolism of other heme containing substances, mainly from cytochromes (Levitt and Levitt 2014). Bilirubin is considered the main part of bile and is responsible for its characteristic coloration (Sticova and Jirsa 2013). The bile pigments bilirubin and biliverdin are endogenous compounds and are part of the porphyrin family (Bulmer, Ried et al. 2008). Furthermore, both show a unique and differing three dimensional structure. The concentration of bilirubin in serum is affected by various factors such as cigarette smoking, intake of different drugs or plant products, gender, ethnicity, age, fasting as well as altitude. All these factors are likely to contribute to the biological effect of bilirubin (Vitek 2012).

The physiological concentration of circulating bilirubin in humans is about 5-17µM and almost all of it is present in the unconjugated fraction bound to albumin (McDonagh 1979). Plasma concentrations higher than >300µM are associated with the risk for neurological disorders (Stocker 2004). However, already concentrations up to 100µM show toxic effects on cell functions.

Toxicity of bilirubin occurs when the molar concentration of bilirubin in the plasma exceeds that of plasma albumin. However, mildly increased levels (>17.1 µM) of bilirubin on the other hand seem to be cytoprotective (Wang, Chowdhury et al. 2006).

Neurotoxicity of bilirubin is referred to the non-albumin-bound (free) part of unconjugated bilirubin (UCB). Predominantly, this fraction is present as a protonated diacid which is capable of passing through cell membranes (Roy-Chowdhury and Roy-Chowdhury 2012), accounting for its toxicity.

2.2.1 Bilirubin structure and chemistry

Bilirubin is part of a phylogenetically old superfamily of tetrapyrrolic compounds and exhibits multiple biological functions (Vitek 2012). Characteristic for the structure of bilirubin are the four covalently linked pyrrole rings in an open linear tetrapyrrolic chain (Tomaro and Batlle 2002). Additionally, bilirubin possesses six internal hydrogen bonds which are accountable for its chemical properties, including poor aqueous solubility, and biological effects (Vitek and Ostrow 2009).

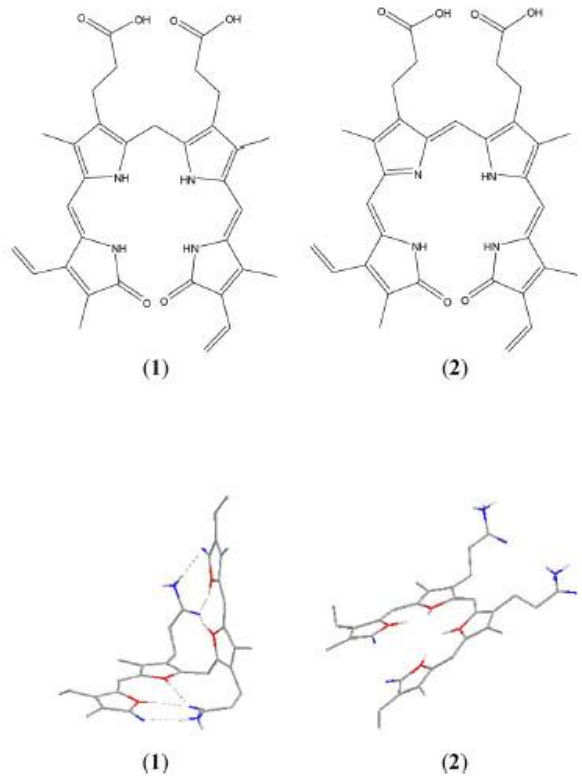

Figure 4. Two- and three-dimensional structures of bilirubin (1) and biliverdin (2) (Bulmer, Ried et al. 2008).

2.2.2 Heme Catabolism

A major metabolic process inside mammalian cells is the degradation of heme which is derived from hemoproteins like cytochrome P450s and hemoglobin (McDonagh 1979). The enzymatic process of heme oxygenase (HO) is considered as the rate-limiting step in the breakdown of heme and results in the generation of equimolar amounts of biliverdin, free iron and carbon monoxide (Abraham and Kappas 2008).

Biliverdin is subsequently reduced, at the expense of NADPH, to unconjugated bilirubin (UCB, indirect bilirubin) by the cytosolic enzyme biliverdin reductase (BVR) (Schmid and McDonagh 1975). Due to its internal hydrogen bonds bilirubin is poorly soluble in water (McDonagh 1979). Therefore bilirubin is tightly bound to albumin for transport in blood circulation (Brodersen 1980).

In the liver glutathione-S-transferase (GSTs) is responsible for the uptake and the intermediate storage of bilirubin in the cytoplasm of hepatocytes. Under physiological conditions, about 98–99 % of bilirubin is conjugated (direct bilirubin) by the enzyme uridine-diphospho-glucuronate-glucuronyl transferase (UGT1A1). After conjugation bilirubin is soluble in water and can be released into the bile (Tomaro and Batlle 2002). Conjugated bilirubin reaches the intestine, is converted to urobilinogens and urobilins and is finally eliminated with the stool (Stocker 2004).

UGT1A1 is the main UGT isoform and accountable for bilirubin glucuronidation. Any impaired or reduced activity of it may results in different forms of unconjugated hyperbilirubinaemia (Wang, Chowdhury et al. 2006).

The direct bilirubin together with the indirect bilirubin represent the total serum bilirubin (0.1–1.2mg/dL total bilirubin, 0.1–0.3mg/dL direct bilirubin) (Jansen and Bittar 2004).

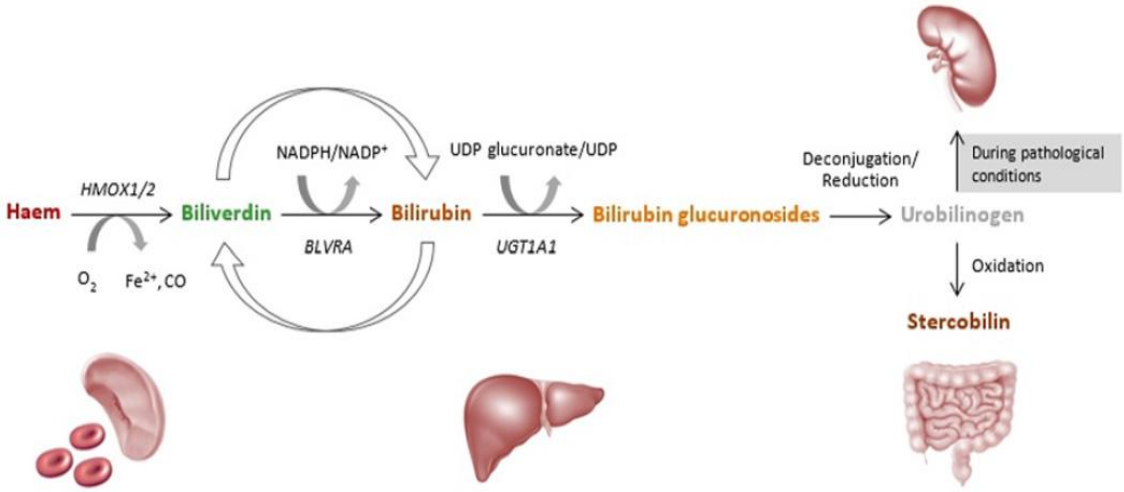

Figure 5. Heme catabolism (Wagner, Wallner et al. 2015).

2.2.3 Hyperbilirubinaemia

Hyperbilirubinaemia may result either from increased bilirubin production or abnormalities in one of the four major steps of hepatic bilirubin metabolism. The crucial steps are the uptake by hepatocytes, the intracellular binding/storage, the conjugation and finally the excretion into the bile (Roy-Chowdhury and Roy-Chowdhury 2012). A reduced uptake of bilirubin from the blood circulation leads to increased levels of unconjugated or conjugated bilirubin in serum/blood (Erlinger, Arias et al. 2014).

In this thesis is focused on the hyperbilirubinaemic condition Gilbert´s Syndrome. Additionally, the severe condition of Crigler-Najjar syndrome (CNS) will be discussed briefly because both originate from the same polymorphism in the promoter region of the UGT1A1 gene.

2.2.4 Crigler-Najjar syndrome (CNS)

Crigler-Najjar syndrome type 1 and 2 is the most deleterious condition of disrupted bilirubin glucuronidation and defined by an absent or strongly diminished UGT1A1 activity in the liver. The CNS type 1 is characterized by a complete absence of bilirubin glucuronidation whereas in type 2 the UGT1A1 activity is decreased to below 30% (Strassburg 2010). The prevalence of CNS is rare and affects $1:10^6$ people worldwide (Nowicki and Rainer Poley 1998). UCB levels can rise up to toxic concentration of 500µM. If such high levels remain untreated and surpass the binding capacity of albumin, unbound (free) UCB can transfer into cellular compartments, especially into the brain. The toxic effect of the underlying mechanisms is not completely investigated. It is generally accepted that bilirubin decreases the mitochondrial membrane potential and introduces ER stress which may result in increased apoptosis. In order to decrease toxic levels of bilirubin in CNS, subjects depend on a life-long phototherapy, which breaks down the bilirubin molecule to yield readily water-soluble lumirubin. If bilirubin rises to extremely high concentrations patients must receive a plasma exchange transfusion. Even liver transplantation is considered, clearly reflecting the severity of this syndrome (Bulmer, Verkade et al. 2013).

2.2.5 Gilbert´s syndrome (GS)

GS is a benign condition of hyperbilirubinaemia and approximately 3–10% of the general population is affected. Although not all of the subjects affected have a clear family history, genetic studies reveal an autosomal-recessive inheritance pattern of GS syndrome (Bosma, Chowdhury et al. 1995).

The genetic background is based on a polymorphism located in the promoter region of the UGT1A1 gene and results in decreased expression of the hepatic enzyme UGT1A1. Hence, bilirubin conjugation and excretion into the bile is reduced. This results in mildly and chronically increased serum UCB levels exceeding 17.1µmol/L in GS subjects (Strassburg 2008).

The activity of the essential hepatic UGT1A1 for excretion of bilirubin is reduced to 30% compared to the activity of subjects without GS (Bosma, Chowdhury et al. 1995).

In the corresponding gene the TATAA element is usually $A(TA)_6TAA$. The majority of Caucasians with GS exhibit 7 TA tandem repeats $A(TA)_7TAA$ in each of the two alleles (homozygous 7/7). The phenomenon of UGT1A1 genes with this modification in the TATAA element is called UGT1A1*28 polymorphism (Roy-Chowdhury and Roy-Chowdhury 2012).

It was demonstrated that individuals with the homozygous genotype 7/7 (UGT1A1*28) had significantly higher bilirubin levels compared to the levels of individuals with the genotype 6/7 and 6/6 (wild-type) (1.14–0.44mg/dL vs. 0.69-0.27mg/dL) (Lin, O'Donnell et al. 2006).

When men and women were considered it was shown that male subjects had higher levels of total serum bilirubin compared to females (0.72+/-0.004mg/dl vs. 0.52+/-0.003mg/dl). Further it was demonstrated that bilirubin concentrations varied significantly between sexes and ethnical groups, and are strongly influenced by lifestyle factors such as smoking (Zucker, Horn et al. 2004).

Furthermore, it is suggested that GS is linked to impairment in the hepatic uptake of bilirubin achieved by organic anion transporters (Strassburg 2010). The hepatic glucuronidation plays an important role in the elimination of a broad range of endogenous and exogenous substrates. Therefore, GS subjects are more likely to be affected by adverse effects of drugs which are metabolized via UGT1A1, namely atazanavir, indinavir or irinotecan (Sticova and Jirsa 2013).

Prevalence of GS is higher in males than in females (Roy-Chowdhury and Roy-Chowdhury 2012). The diagnosis of GS can be conducted after a 24h-fast. When the caloric intake within this period does not exceed 400kcal, the UCB levels of GS subjects could be doubled. Such an increase is neither observed in healthy persons nor seen in patients with hemolysis and liver diseases

(Owens and Sherlock 1973). Additionally, liver functions and blood cell concentrations need to be in a normal range.

2.3 Protective role of bilirubin

2.3.1 Bilirubin as antioxidant

Bilirubin the lipid-soluble degradation product of heme catabolism in mammalian cells, was generally considered as cytotoxic waste product that needed to be excreted However, over the past 20 years studies have demonstrated that bilirubin possesses multiple biological properties such as antioxidant effects (ROLAND STOCKER and ALEXANDER N. GLAZER I987), anti-inflammatory effects (Wallner, Bulmer et al. 2013) and anti-carcinogenic properties (Ollinger, Kogler et al. 2007, Molzer, Pfleger et al. 2013).

A study from the year 1954 provides indirect evidence for the anti-oxidative activity of bilirubin, along with biliverdin. It has been noted that even small amounts of bile stabilize vitamin A and ß-carotene during the absorption in the intestinal tract and prevent lipids from oxidative attacks, particularly linoleic acid (Bernhard, Ritzel et al. 1954).

The anti-oxidative activity of bilirubin can be explained by its chemical structure. All physiologically abundant fractions of bilirubin have common features: an extended network of conjugated double bonds (pi-eclectrons) and a pair of reactive hydrogen atoms (H) (Stocker 2004).

The reaction can be described as follows: LOO• + Bilirubin → LOOH + Bilirubin•

In vitro studies indicate that unconjugated bilirubin (UCB) efficiently scavenges peroxyl radicals (ROO•) in homogenous solution or in multilamellar liposomes. Its anti-oxidative potential is increased under physiological conditions of low oxygen tension and even capable to surpass the anti-oxidative potential of α-tocopherol. The water-soluble conjugated fraction of bilirubin is similarly efficient in preventing lipids from peroxyl radical induced oxidation (Stocker 2004).

Also the albumin–bound bilirubin fraction (at concentrations present in human plasma of healthy adults) acts as potent peroxyl radical scavenger and furthermore prevents fatty acids, which are transported via albumin through the entire circulation, from oxidative damage (Stocker, Glazer et al. 1987).
Along with the direct scavenging activity of bilirubin towards peroxyl radicals also the synergistic effect of bilirubin and biliverdin with membrane-bound α-tocopherol, the major lipid-soluble antioxidant in humans, is worth mentioning. Whilst the absence of α-tocopherol results in an ongoing process of lipid peroxidation within the liposome membranes, the presence of low amounts of bilirubin has the potential to inhibit this oxidation (Neuzil and Stocker 1994).
Binding of bilirubin to albumin enhances the reactivity and specificity against reactive oxygen and nitrogen species (RONS). This results in an increased protection of plasma proteins and lipids and arises from bilirubin´s potential to scavenge both 1e- and 2e-oxidants, such as singlet oxygen, hypochlorous acid, quinines and peroxynitrite. Many other antioxidants are only effective against one mechanism of oxidant action (Stocker 2004).

In vitro experiments have indicated that a bilirubin concentration of 10µM is enough to cope with a 10.000-fold higher dose of hydrogen peroxide radicals and prevent adverse effects to cells. A possible explanation for this efficient action is provided through the biliverdin-bilirubin antioxidant cycle or more precisely through the redox recycling of bilirubin. If bilirubin scavenges toxic peroxyl radicals in cells, it gets oxidized itself to yield biliverdin. Subsequently, biliverdin is recycled back by BVR to bilirubin. This regenerative recycling of bilirubin amplifies the protective effect of bilirubin at micromolar concentration. The recycled bilirubin is able to act as antioxidant over and over again (McDonagh 2010). It is noteworthy, that this explanation is speculative at this time and further investigations are needed in this matter.

After the *in vitro* antioxidant activity of bilirubin was discovered, a series of epidemiological studies followed, to explore possible protective effects *in vivo*.

These studies focused on assessing the potential role of bilirubin as an anti-mutagenic, anti-oxidative and anti-atherogenic molecule in the prevention of chronic, so called non-communicable diseases (NCDs).

NCDs represent 63% of the total number of deaths worldwide and oxidative DNA damage plays a crucial part in the development of NCDs (Milic, Frustaci et al. 2015).

Therefore it is of great importance to explore whether there is a connection between subjects presenting moderately elevated bilirubin levels and a possibly reduced risk for developing CVDs and cancer. Corresponding studies are presented in the following paragraphs.

2.3.2 DNA damage and repair in Gilbert´s syndrome subjects

Oxidative stress can lead to severe oxidative damage to the double helix of DNA. Unrepaired lesions in the DNA lead to genomic instability which in turn might be the reason for mutations and carcinogenic processes.

A study examined the DNA damage in GS and control subjects and could not observe differences in the cellular level of DNA damage between the two groups. Although, they provided noteworthy findings in regard to the repair capacity in hyperbilirubinemic rats (Gun rats): a higher level of strand breaks were observed which might reflect a higher DNA damage occurring or possible a higher incision activity of repair enzymes which introduce SSBs (Wallner, Antl et al. 2013).

The aim of the study performed by Wallner et al. was to observe the association between moderately elevated serum bilirubin concentrations and the level of chromosomal and cytological DNA damage by using the cytokinesis-block micronucleus cytome assay (CBMN) and the buccal micronucleus cytome assay (BMcyt). 76 subjects were enrolled in the study and allocated, based on their serum bilirubin concentration, into the GS group (UCB ≥17.1µM) or into the control group (UCB >17.1µmol). Significant differences of CBMN and

BMcyt endpoint parameters between the two groups could not be shown. Nevertheless, interesting results were provided for GS subjects. The formation of buccal micronucleated cells and buccal nuclear buds were lower in older (≥35 years) GS subjects compared to younger (<30 years) GS subjects which indicates a protection against changes in the genetic material for older subjects with GS (Wallner, Blassnigg et al. 2012).

A randomized, controlled study used the comet repair assay to evaluate the relation between UGT1A1*28 polymorphisms and DNA damage and DNA repair parameters. Results indicate no association between DNA damage and the UGT1A1*28 variant. However, individuals with the genotype UGT1A1*28 show a decreased capacity of DNA repair. The study population however was small and comprised only of 17 women and 11 men, which is a limitation of this study (Chang, Chen et al. 2010).

2.3.3 Bilirubin, GS and cardiovascular diseases (CVDs)

CVDs are worldwide the main reason (80%) of mortality in adults that are older than 65 years (Karavidas, Lazaros et al. 2010). It is suggested that oxidative stress supports endothelial dysfunction, atherosclerosis and inflammatory diseases of the arterial wall that in turn leads to chronic and acute cardiovascular processes (Locatelli, Canaud et al. 2003).

Bulmer et al conducted a study and investigated the mechanism behind bilirubin´s protective effects against CVDs. Subjects with GS (UCB >17.1µmol/L; 1mg/dL) had a reduced frequency of CVDs. 9 individuals having GS were matched with 12 normobilirubinemic control individuals. GS subjects had an increased antioxidant status and a reduced susceptibility to serum oxidation which as a consequence might have led to the reduced prevalence of CVDs observed in people with GS (Bulmer, Blanchfield et al. 2008). Furthermore it was pointed out that a decreased LDL status in GS subjects is a predictor variable for decreased oxLDL and in turn a valuable biomarker for the risk of CVDs (Bulmer, Verkade et al. 2013).

In a retrospective study it was demonstrated that a reduction in the total serum bilirubin concentration seems to be a predictor for the incident of LDL cholesterolemia in healthy subjects (Oda 2014).

Furthermore, a group of investigators observed the relationship between lipid status and mildly elevated blood bilirubin concentration (>17.1µmol), as seen in GS subjects. They observed a significantly improved ($p<0.05$) lipid status in GS subjects when compared to a gender- and age matched controls. The concentrations of total cholesterol, LDL-cholesterol, triacylglycerol (TAG), pro-atherogenic LDL subfraction and Apo-B were reduced in GS subjects (Wallner, Marculescu et al. 2013).

Tapan et al. evaluated the role of lipid parameters (small dense low-density lipoprotein cholesterol sc-LDL-C, ox-LDL) as well as markers of inflammation (high-sensitive C-reactive protein, hs-CRP) in GS subjects. The study population consisted of 42 subjects with GS compared to 52 healthy controls. Those lipid parameters considered, were reduced in GS subjects, as compared to controls. This led to the conclusion that the reduced lipid status observed, might be a reason for lower risk of artheriosclerosis in individuals with GS (Tapan, Karadurmus et al. 2011).

In terms of artheriosclerosis it has been pointed out that an elevation of 1µmol/L in serum bilirubin results in a 14% reduced likelyhood for having a CAC score higher than 400. The CAC score is a valuable marker for coronary atherosclerosis. This strong relationship was shown in men and in women (Tanaka, Fukui et al. 2009).

A retrospective 24-years follow up study was carried out and a subset of 1,780 males (mean age of 36 years) were included. In this study, the UGT1A1*28 polymorphism statistically reduced the risk for CAD and CVD by two-thirds (Lin, O'Donnell et al. 2006).

Further research in the development of coronary heart diseases has been completed by Vitek et al. In 111 male GS subjects it was shown that the progression of intima-medial thickness (IMT) of carotid arteries was significantly

delayed compared to subjects with normal bilirubin levels (Vitek, Novotny et al. 2006). Further examination by Vitek et al. showed that the prevalence of ischemic heart disease (IHD) in subjects with GS is 2% compared to 12% within the general population. Additionally, the total antioxidant capacity (TAC) and HDL concentration were significantly higher in GS individuals (Vitek, Jirsa et al. 2002).

The large Framingham Heart study (FHS) was performed in the year 1948 as a prospective analysis of the risk for CVDs in a population based cohort. In one of the many Framingham Offspring studies the relations between elevated serum bilirubin and myocardial infarction (MI), coronary death and different cardiovascular events (congestive heart failure, intermittent claudication and stroke) were assessed. To this end, samples of 4,276 middle aged participants of both genders were analyzed for bilirubin, and results were correlated to disease markers. The study authors concluded that increased total serum bilirubin levels are linked to a lower risk for MI, CAD and cardiovascular events in men. These findings could not be confirmed in women. It was suggested that women might be less susceptible to decreased levels of bilirubin due to lifestyle factors (higher intake of anti-oxidative vitamins A, C and E) or biology (Djoussé, Levy et al. 2001).

To sum up the studies, the protective role of bilirubin in the development of CVDs might be due to its anti-oxidative, anti-inflammatory and lipid-lowering potential. To date, definite mechanisms of action on a molecular level are elusive. Therefore, bilirubin is considered a correlated biomarker, based on a large number of epidemiological and clinical observation studies.

2.3.4 Bilirubin, GS and cancer

Bilirubin has strong anti-oxidative potential *in vitro* and *in vivo*, and could thereby lower oxidative processes and possibly prevent cancer in humans.

The aim of a Czech case-control study was to assess the association between the UGT1A1*28 promoter variation and the serum bilirubin concentration with

the risk for CRC. Genetic analyses were conducted in 777 CRC cases and 986 healthy controls which were all part of the same geographic region. Respectively, a 20% decreased risk for CRC was observed in the UGT1A1*28 allele carrier. According to gender, the protective effect was primarily observed in males and less pronounced in females. In a subset of 174 CRC cases and 247 controls it was shown that CRC patients had significantly lower levels of serum bilirubin as compared to healthy controls (9.8µmol/L vs.11.35µmol/L). Each 1µmol/L reduction in serum bilirubin was related with a 7% elevated risk for CRC. Again, correlations were more pronounced in men than in women (Jiraskova, Novotny et al. 2012).

Similar evidence was provided by Zucker et al. They observed in a cross-sectional study of nearly 17,000 subjects the correlation between bilirubin concentrations and the prevalence of CRC. An inverse correlation between total serum bilirubin and a history of CRC was observed (Zucker, Horn et al. 2004).

A large longitudinal cohort up study including 218,727 men and 285,479 women from the UK was conducted for 8 years to examine the connection between total serum bilirubin concentrations and the incidence of lung cancer. The results indicate that an increase of the total serum bilirubin concentration of 0.1mg/dl decreases the incidence of lung cancer (8% decline in men, 11% in women) (Horsfall, Rait et al. 2011).

Temme et al. examined in a large 10 year follow-up study the relation between serum bilirubin concentrations and all-cause, cardiovascular and cancer mortality. 5,460 men and 4,843 women were included in the study. Results indicated that men with higher serum bilirubin levels (≥10.3µmol, >=0.6mg/dl) in contrast to those with lower levels (<3.4 µmol, <=0.2mg/dl) had a lower relative risk (RR) for all-cause mortality (RR=0.73) and cancer mortality (RR=0.42). A significant inverse correlation between serum bilirubin and the risk for cancer mortality was observed in men, especially concerning the mortality of non-lung cancer types. Statistical significance was lacking in women (Temme, Zhang et al. 2001).

Studies which investigate the relationship of serum bilirubin concentrations and breast cancer provide different results for women:

The aim of the study performed by Ching et al. was to assess the association between micronutrient and antioxidant status with the risk for breast cancer. To this end, blood samples of 153 women having breast cancer and those of 151 healthy women were screened. Serum albumin, bilirubin, uric acid and the total antioxidant status relative to trolox were determined. Females having higher bilirubin levels (≥7.5µM) compared to those with lower levels (≤4.1µM) showed a decreased risk for breast cancer (Ching, Ingram et al. 2002).

Guillemette et al. investigated in a nested case-control study the relationship between the UGT1A1*28 allele ($A(TA)_7TAA$) (the main underlying genotype of GS among caucasians), and the risk for breast cancer. The study population involved 461 women with incident breast cancer, and 615 matched controls. DNA samples were genotyped for the UGT1A1 polymorphism. No significant association between the UGT1A1*28 allele and breast cancer was determined (Guillemette, De Vivo et al. 2001).

The underlying mechanisms of possible anti-mutagenic properties (in breast and other cancers) of bilirubin are not completely understood and further investigations are needed.

Bilirubin has shown in a dose-dependent way the anti-proliferative effects in various tumor cell lines, as a consequence of interrupting cell-cycle progression, and initiating apoptosis. Furthermore, bilirubin interferes with a few pro-carcinogenic signal molecules, such as p53, p27, caspase 7, PARP or ERK. Bilirubin concentrations required for those anti-proliferative effects on colon cancer cells observed, are similar to those of serum bilirubin levels naturally abundant in humans, which is why bilirubin might also act as efficient cancer preventing agent *in vivo* (Ollinger, Kogler et al. 2007).

Indications for bilirubin´s potential to decrease the viability of colon adenocarcinoma cell lines have been provided by an *in vitro* study. It has been suggested that bilirubin induces apoptosis in rat brain neurons which may be

explained by the mitochondrial (intrinsic) apoptosis pathway. Further evidence suggests that bilirubin initiates mitochondrial depolarization in colon cancer cells and triggers apoptosis in human gastric cancer cells (Keshavan, Schwemberger et al. 2004).

Mölzer et al. analyzed whether different tetrapyrroles were able to induce DNA damage to cultured human cancer cell lines (Caco2, HepG2). DNA damage was assessed with the SCGE. They demonstrated that most of the investigated tetrapyrroles were capable to provoke DNA damage in Caco2 and HepG2 cells and this was especially seen for intestinal tetrapyrroles such as stercobilin, urobilin and protoporphyrin (Molzer, Pfleger et al. 2013).

The above *in vitro* anti-genotoxic observations could therefore explain the reduced prevalence of cancer *in vivo*. However, further research for better understanding the complete underlying mechanism of action of bilirubin should be conducted.

3 MATERIALS AND METHODS

3.1 Study design and inclusion/exclusion criteria

In the present case-control study a total number of 128 subjects were included. The subjects were recruited using information-sheets posted at universities, hospitals, and pharmacies. Participants were well informed about the study procedure and were refunded for their attendance.

The total study population was divided into two groups (case and control) and assignment to one of the two groups was based on the subject´s serum unconjugated bilirubin concentration. The unconjugated serum bilirubin concentration was determined by high-performance liquid chromatography (HPLC). Seven subjects had to be excluded due to unclear UCB results (genotype information was not available at this time). 61 persons were identified as GS subjects and they exhibited UCB levels ≥17.1µM (≥1mg/dl) and were defined as the GS group. 60 subjects were allocated to the control group and their UCB levels were lower than <17.1µM (<1mg/dl). The two groups were matched for gender and age. The study design is summarized in figure 6.

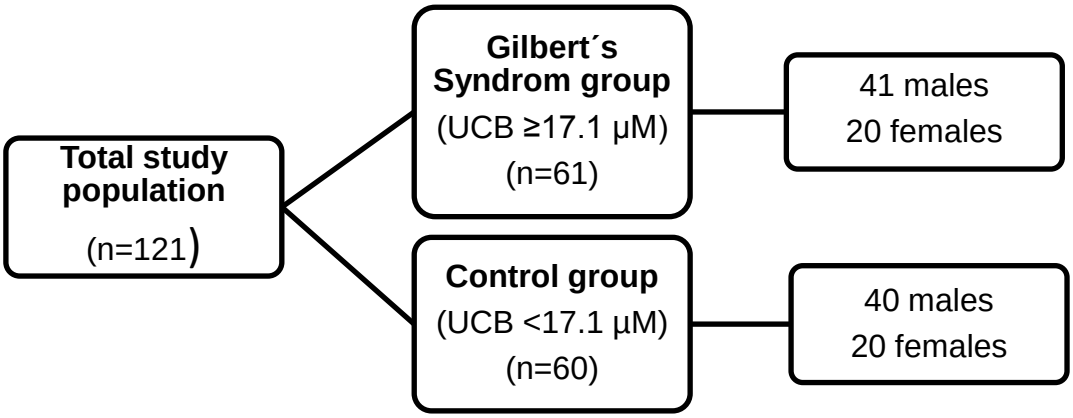

Figure 6. Study design

Two visits to the General Hospital of Vienna (AKH) were required for participation. At the screening appointment subjects had to sign an informant consent. Thereafter a small blood sample was taken and questionnaires had to be completed to check for inclusion and exclusion criteria. Included subjects were advised to consume not more than 400kcal the day before the main blood sampling appointment. Subsequently, after the second and main visit, samples were transported to the Department of Nutritional Science for further analyses such as the *in vitro* Comet repair assay. With the *in vitro* Comet repair assay, we observed whether subjects with GS compared to control subjects have a better capability for repairing oxidatively-damaged DNA. The study was approved by the Ethical Committee of the Medical University of Vienna and the General Hospital of Vienna (#1164/2014) and conducted according to the Declaration of Helsinki.

Inclusion criteria

- Age: 20–80 years
- total serum bilirubin for GS subjects: >20.52µmol/L (>1.2mg/dl)
- unconjugated serum bilirubin for GS subjects: >17.1µmol/L (>1mg/dl)
- total serum bilirubin for control subjects: <20.52µmol/L (>1.2mg/dl)
- unconjugated serum bilirubin for control subjects: <17.1µmol (<1mg/dl)
- blood gamma-glutamyltranspeptidase (g-GT) <100IU
- blood alanin-aminotransferase (ALAT) <100IU
- blood aspartat-aminotransferase (ASAT) <100IU
- physical activity (moderate)

Exclusion criteria

- Age: <20 years or >80 years
- cardiovascular diseases
- hepatitis B/C or any other liver diseases
- cholelithiasis
- hemolysis
- chronic kidney diseases

- past or present cancer
- diabetes mellitus
- supplementation with antioxidants in the last four weeks before the first blood sampling
- medications which influence liver parameters (e.g. Probenecid, Rifampicin)
- patients with organ transplants
- smokers
- alcohol intake, more than 7 drinks per week
- competitive athletes, more than 10 hours workout per week

3.1.1 Sterile isolation of peripheral blood mononuclear cells (PBMCs)

The isolation of PBMCs was done by using a laminar flow which maintained sterile conditions. PBMCs were isolated from the human study samples. Furthermore, PBMCs were isolated from one healthy subject and used for the substrate preparation.

- Venous blood was collected by anticoagulant EDTA tubes.
- Tubes were carefully pivoted 8 times from upside down.
- Blood in the EDTA tubes was transferred to ficoll-containing Leucosep tubes (Greiner Bio-one). Clear separation had to be maintained between the ficoll medium and the blood layers (precise and fast working is important). Leucosep tubes which are stored at 4°C had to be used at room temperature.
- Tubes were centrifuged at 3,000rpm (1,300xg) for 15 minutes at room temperature, without brake. Blood fractions were separated according to their density.
- The plasma fraction was removed, chilled and stored for further analysis.
- Buffy coat (enriched PBMCs) was carefully harvested into a 50ml centrifuge tube and filled up with cold sterile PBS. Further procedure was done on ice.

- The PBMC suspension was centrifuged at 3,000rpm, 10 minutes at 4°C, with brake (washing step).
- Supernatant was removed, PBMCs were resuspended and washed again by filling up approximately half with cold sterile PBS.
- Washing step was repeated and the remaining cell pellet was resuspended in 5ml PBS for cell counting.

3.1.2 Cell counting

Cells were counted on an automated cell counter (Countess, Invitrogen). For an appropriate cell count a concentration of $5x10^6$ cells/ml should be reached. All steps were done on ice.

- 20µl of the cell suspension was diluted with 220µl cold, sterile PBS in a small cup (dilution factor 12).
- 10µl of the cell suspension was mixed with 10µl Trypan blue.
- 10µl of this mixture was pipetted in a chamber on a specific slide and inserted into the Countess.
- Cells were counted twice for each sample and the mean value used for further calculations.
- Total cell number was calculated and cell suspension aliquoted. Duplicates of each sample were prepared.

3.1.3 Freezing PBMCs for the *in vitro* Comet repair assay

- Aliquoted cell suspension was centrifuged at 3,000rpm, 10 minutes at 4°C.
- Supernatant was removed and the pellet was resuspended in 1ml buffer A (preparation see page 38).
- Centrifuged at 3,000rpm, 5 minutes at 4°C.
- Supernatant was carefully and completely removed from the cell pellet.
- Cell pellet was snap-frozen with liquid nitrogen and stored at -80°C.

3.2 *In vitro* Comet repair assay

The aim of the *in vitro* Comet repair assay is to quantify DNA repair incision activity. A DNA substrate (cells from one healthy person) was prepared. The cells of the substrate were pre-treated with a photosensitiser (Ro 19-8022) plus light (Tungsten halogen lamp, Brennenstuhl) in order to create oxidative base lesions (8-oxoG). The *in vitro* induced cell damage is specific for the pathway of interest. In our case, the repair pathway, BER, was studied which is primarily responsible for the repair of oxidative base lesions. The substrate cells were lysed and nucleoids were formed. The extract (human study samples) with the repair enzymes of interest was incubated with the damaged substrate. If the human repair enzymes (hOGG1) recognize the oxidized base lesions, DNA becomes incised and single strand breaks (SSBs) occur. SSBs can be analyzed with alkaline gel electrophoresis where they migrate towards the anode and form a typical comet tail. In fact, increased % DNA in tail reflects the repair incision activity of the repair enzymes in the study samples (Azqueta, Langie et al. 2013).

3.2.1 Equipment for the *in vitro* Comet repair assay

Material	Company	Product number
Analytic balance (0.01mg–220g)	Mettler	AT201
Centrifuge	Eppendorf	5417R
Countess-automated cell counter	Invitrogen	C10227
Countess-cell counting chamber slide	Invitrogen	C10283
Drying chamber	Memmert	Modell 500; D06061
Electrophoresis tank (0-200V; 0-100mA)	PeqLab	41-2325-R
Electrophoresis Power supply	PeqLab	EV231
Fluorescence microscope (20x/0.40)	Nikon	Eclipse Ci-L
Heating Plate/Magnetic stirrer	Heidolph	MR 3001K
Lumen 200 (Lamp for fluorescence microscope)	Prior	L200D
Microscope Cover glasses (24x32mm)	VWR	631-1572
Microscope slides	VWR	ECN 631-1551
Microwave 320V, 50Hz, 700W	Elta	MW 170 (17MXS1);
pH meter	Metrohm	827 pH Lab
Pipette 5µl–50µl	Biohit	8103949
Pipette 20µl–200µl	VWR	259050316
Pipette 100µl–1,000µl	VWR	259060722
Scale	Sartourius	LC 480 1P-OCE
Tungsten halogen lamp (230V/max. 500W)	Brennenstuhl	PL 500

Material	Company	Product number
Water bath (37°C, 55°C)	GFL	89585
Moist box		
Multichannel pipette		
12-Gel comet assay units		

Table 1. Equipment for the in vitro Comet repair assay

3.2.2 Reagents for the *in vitro* Comet repair assay

Reagents	Company	Product number
Bovine Serum Albumin (BSA)	Sigma Life Science	A2153-50G
Bradford Reagent	Sigma Life Science	B6919
Dithiotheitol	Sigma Life Science	D0632
Dulbecco´s Phosphate buffered	Sigma Life Science	D5837
Saline (PBS)		
Ethanol	Merck	603-002-00-5
Ethylenediamine tetraacetic acid (EDTA)	Sigma Life Science	E6758
Formamidopyrimidin-DNA	Biolabs	0061405
Glykosylase (FPG)		
Glycerol 99%	Sigma Life Science	G901-2
GelRed Nucleic Acid Gel Stain	Biotium	41003
Liquid Nitrogen		
Photosensitizer RO 19-8022		
Potassium Chloride (KCL)	Merck	49.360.500
Potassim Hydroxic >86% (KOH)	Sigma Life Science	60370

Reagents	Company	Product number
Sodium Chloride (NaCl)	Sigma Life Science	7647-14-5
Sodium hydroxide pellets (NaOH)	Sigma Life Science	1310-73-2
Triton-X-100	Sigma Life Science	9002-93-1
Trizma base (Tris)	Sigma Life Science	77-86-1
Trypan blue stain 0.4%	Invitrogen	761866
Ultra Pure Agarose (NMA)	Invitrogen	16500-100
Ultra Pure LMP Agarose (LMA)	Invitrogen	16520-050

Table 2. Reagents for the in vitro Comet repair assay

3.2.3 Preparation and storage of solutions for the *in vitro* Comet repair assay

Almost all solutions were prepared and stored at -20°C a few weeks before the Comet repair assay was performed. A few ones had to be prepared fresh.

Alkaline electrophoresis solution (pH>13)

NaOH (0.3M) 24.0g

EDTA (0.001M) 0.58g

The electrophoresis solution had to be prepared fresh on the day of Comet repair assay. Both reagents were dissolved in 2l bi-distilled water. The pH had to be greater than 13 (checked with pH meter). The electrophoresis solution was stored at 4°C.

Lysis solution (pH=10)

NaCL (2.5M) 146.1g

EDTA (0.1M) 29.2g

Tris base (10mM) 1.211g

Chemicals were dissolved in 1l bi-distilled water. A pH of 10 was adjusted with NaOH (10M) and stored at 4°C. Lysis solutions are stable for one week. Shortly before usage 1ml Triton X-100 was added to the lysis solution (100ml) and mixed carefully on the magnetic stirrer.

Buffer F (negative control and washing buffer after lysis)

HEPES (40mM) 95.3g

KCL (0.1M) 74.56g

EDTA (0.5mM) 1.46g

BSA (0.2mg/ml) 2g

Reagents were dissolved in 1l bi-distilled water and adjusted to a pH of 8 with KOH (1M). We prepared 40ml aliquots and stored them at -20°C. At the day of the Comet repair assay one 40ml aliquot was thawed in a water bath and filled up to 400ml with bi-distilled water. The pH was checked and when necessary adjusted again to pH of 8 with KOH (1M).

FPG (positive control)

FPG 10µl

Buffer F 290µl

The FPG stock solution was used at a 1:3,000 dilution. 10µl of the FPG stock solution was mixed with 290µl buffer F (1:30). We prepared aliquots of 15µl and stored them at -20°C. At the day of Comet repair assay the 15µl FPG aliquot was thawed and filled up with 1485µl buffer F (1:100), carefully mixed and placed on ice until usage.

Buffer A (extraction buffer, freezing medium)

HEPES (0.045M)	1072mg
KCL (0.4M)	2982mg
EDTA (0.001M)	292mg
Dithiothreitol (0.0001M)	20mg
Glycerol 10%	15g (≈ 12ml)

Chemicals were dissolved in 100ml bi-distilled water and adjusted to pH of 7.8 with KOH (6M). Aliquots of 0.5ml were prepared and stored at -20°C.

Buffer A with Triton X-100 (1%)

The buffer A solution was prepared again (see above). Additionally, 1ml of Triton X-100 was added to 100ml buffer A. Again, aliquots of 0.5ml were prepared and stored at -20°C.

Freezing Medium

The buffer A was also used as freezing medium. 100ml of buffer A solution was prepared, adjusted to pH 7.8 with KOH (6M) and stored in 5ml aliquots at 20°C. When PBMCs were isolated and frozen for the *in vitro* Comet repair assay one aliquot (5ml) was thawed and filled up to 15ml with bi-distilled water (1:3).

Normal melting point agarose 1% (NMP; for pre-coating slides)

In the microwave 200mg of NMP agarose was dissolved in 20ml bi-distilled water. Till the date of use NMP agarose was stored in the refrigerator.

Pre-coating slides:

NMP agarose was liquefied in the microwave (800W) and placed in a water bath (55°C) for keeping the solution liquid. Slides were immersed in the agarose solution. The back side was carefully wiped off with a paper towel. Slides were dried over night and stored at room temperature until use.

Low melting point agarose 1 % (LMP; for producing substrate-gels)

200mg of LMP agarose was dissolved in 20ml PBS in the microwave, aliquoted in 10ml small bottles and stored in the fridge.

10M NaOH (molecular weight: 40g/mol)

200g NaOH was dissolved in 500ml bi-distilled water. NaOH pellets were added slowly and in small portions to the water. The solution was stored at a dark place at room temperature.

1M KOH (molecular weight: 56.12g/mol)

28.06g KOH was dissolved in 500ml bi-distilled water and stored at a dark place at room temperature.

Photosensitiser RO 19-8022 (molecular weight: 432.907g/mol)

The preparation of the stock solution (1mM photosensitiser in 70% Ethanol) was done in a dark room. We dissolved 10.82mg of the photosensitiser in 25ml 70% Ethanol. The stock solution was aliquoted into 15µl tubes and stored at -20°C at a dark place. On the day of use (substrate preparation) 8990µl PBS was added to the stock solution.

Gel Red Nucleic Acid Gel Stain

The Gel Red stock solution was diluted with bi-distilled water in a ratio 1:33,000. We prepared a 15ml tube and stored it at 4°C until use.

3.2.4 Protocol for the *in vitro* Comet repair assay

The *in vitro* Comet repair assay was performed according to the protocol of Azqueta et al. (Azqueta, Langie et al. 2013). In the figure below the basic steps are demonstrated. The study samples are referred as extracts in the Comet repair assay.

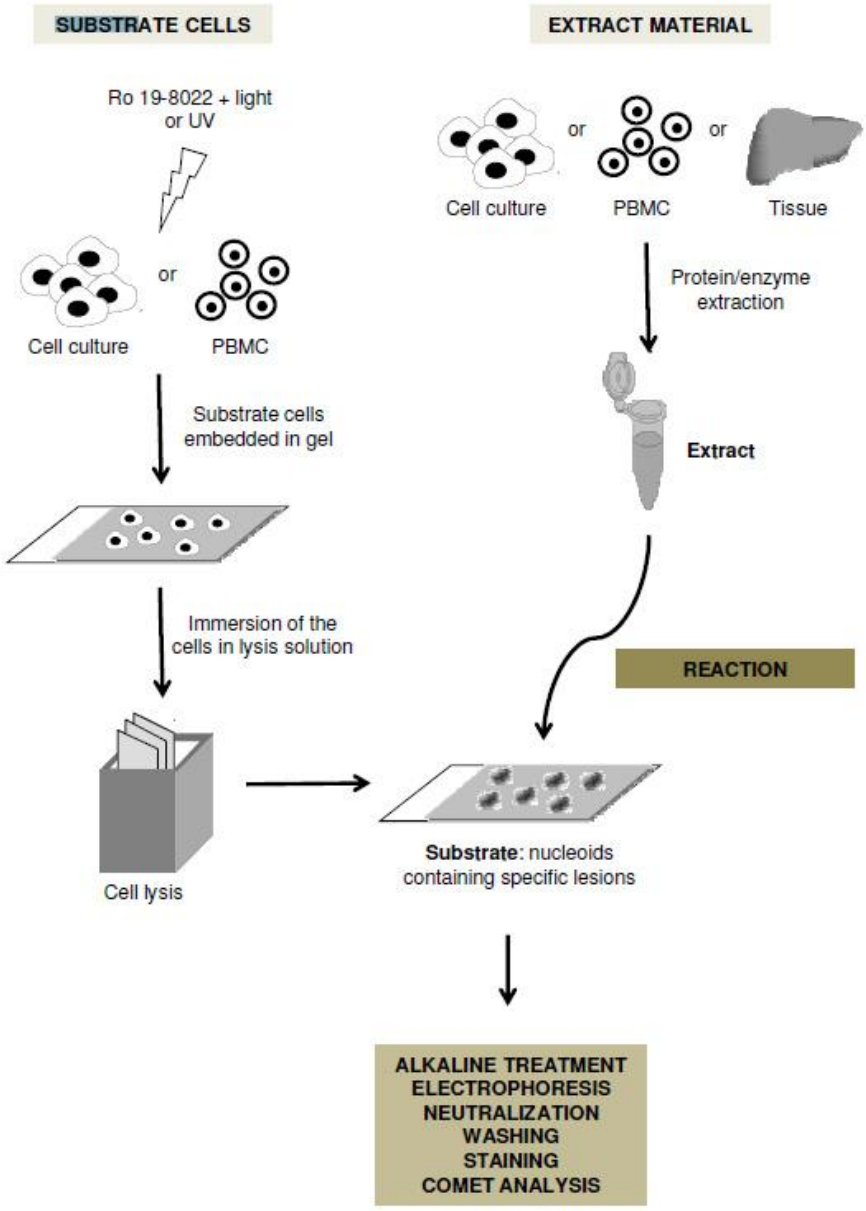

Figure 7. General procedure of the in vitro Comet repair assay (Azqueta, Langie et al. 2013).

Substrate preparation (*in vitro* damaged cells):

For the substrate preparation we isolated PBMCs from venous blood collected from one healthy subject. After isolating and counting we received $5.3x10^6$ living cells per 1ml (protocol for PBMCs isolation and counting see page 32/33). After multiplying the cell count with the dilution factor 4 and the actual used volume of PBS of 10ml we received a total cell suspension of $212x10^6$/10ml. For the substrate preparation we used a cell suspension of $180x10^6$cells/10ml.

- Two 15ml tubes were labeled for RO+ cells (exposed) and two tubes for RO- cells (non-exposed; treatment control).
- Cell suspension ($180x10^6$cells/10ml) was equally divided (2.5ml) in the four prepared 15ml centrifuge tubes.
- Tubes were filled up to 10ml with PBS.
- Further working steps were done in a dark room and excessive light was avoided.
- 10µl of the photosensitiser was added to the two RO+ tubes (not to the RO- tubes).
- Each of the four tubes was carefully transferred to petri dishes.
- Petri dishes were placed on ice exactly 33cm below a 500W halogen lamp and irradiated for 5min. Every 30 seconds the petri dishes were slightly mixed.
- Cell suspensions were completely removed from the petri dishes and pipetted to fresh and labeled tubes (2xRO+, 2xRO-) and were covered with aluminium foil.
- A centrifugion step was done at 1,700rpm, 7 minutes and 4°C.
- Supernatant was removed and pellets resuspended and filled up to 15ml with PBS.
- Tubes were pivoted 5 times from upside down.
- Centrifuged at 1,700rpm, 7 minutes and 4°C.
- Supernatant was removed.
- The two RO+ tubes were combined in one RO+ tube (same for the RO- tubes).

- Pellets were resuspended in PBS and each of the two tubes (RO+ and RO-) was filled up to 5ml with PBS.
- From each cell suspension (RO+, RO-) 20µl were mixed with 60µl PBS (1:4) for cell counting.
- We calculated a total living cell number of $46x10^6$cells/5ml PBS for RO+ and $88x10^6$cells/5ml PBS for RO-.
- We prepared respectively 100 aliquots of each RO+ cells and RO- cells, enough for the whole study. Aliquots contained $5x10^5$ cells.
- Aliquots were centrifuged at 1,700rpm, 5 minutes at 4 °C.
- Supernatant was removed and pellets resuspended in 0.5ml freezing medium (FBS and 10%DMSO).
- Aliquots were slightly frozen by using “Cool cells” and afterwards stored at -80°C for later analyses.

One RO+ and one RO- aliquot was needed for one comet run (sufficient for 10 slides).The preparation of the substrate cells and the Comet repair assay was not performed on the same day. Therefore it was needed to freeze the cells and thaw them when the Comet repair assay was performed.

<u>Thawing the substrate cells (in the dark):</u>

- One RO+ and one RO- aliquot were thawed at 37°C.
- Centrifuged at 4,000rpm, 3 minutes, 4°C.
- Supernatant was removed and cell pellets were resuspended in 0.5ml PBS.
- Centrifuged at 4,000rpm, 3 minutes, 4 °C.
- Supernatant was removed and pellets resuspended in 300µl PBS.
- Cell counts were checked in both tubes (RO+, RO-) and adjusted to a sufficient cell number of $4x10^5$cells.

Place the substrate cells on pre-coated microscope slides (12-gel method):

- 12-Gel comet assay units (metal positioning guide) were put on ice and NMP-agarose pre-coated slides were placed on the metal units.
- Prepared LMP agarose was slowly liquefied by microwave.
- During working LMP agarose was placed in the water bath (37°C). The 10ml bottles with LMP agarose should not be heated more than 3 times.
- 15µl cell suspension of each RO+ and RO- was mixed with 70µl agarose in a tube.
- From each cell suspension 12x5µl dots (a multichannel pipette was used) of the mixture were pipetted on a microscope slide to yield thin minigels.
- Subsequently, slides were placed, back to back, in a cuvette which contained 100ml of the lysis solution. 1ml of Triton X-100 was added to the lysis solution just before use. Slides stayed in there for minimum of 1 hour at 4°C.

In the meantime the extracts (human study samples) the positive- and negative controls were prepared. Totally, ten slides per one comet repair throughput were prepared, five slides with RO+ substrate and five slides with RO- substrate (half of these serve as backup-slides).

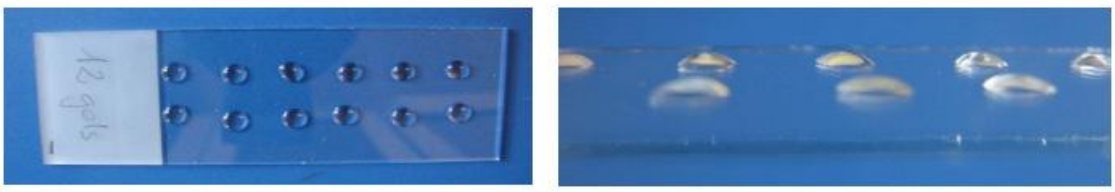

Figure 8. Microscope slide containing 12 minigels (Azqueta, Langie et al. 2013).

Extract preparation (human study samples):

- Ten study samples for one comet repair throughput were used.
- Frozen study samples, 1 aliquot buffer A and 1 aliquot buffer A + 1% Triton-X-100 were thawed at 37°C.
- 50µl of buffer A was pipetted to the extracts.
- Extracts were snap-frozen with liquid nitrogen and thawed again.
- 15µl of buffer A + 1% Triton-X-100 was pipetted to the extracts and thoroughly mixed.
- Extracts were placed on ice for 10 minutes.
- Afterwards, centrifuged at 3,000rpm, 5 minutes at 4°C.
- 60µl of the supernatant of each sample was pipetted into new tubes and gently mixed with 240µl buffer F (1:4).
- Prepared extracts were placed on ice for the further working process.

Preparation of the positive control (FPG):

- One aliquot FPG (15µl) was thawed at 37°C.
- 1,485µl buffer F was added to the 15µl FPG aliquot.
- Thoroughly mixed and placed on ice.

Preparation of the negative control (buffer F):

- 800µl prepared buffer F was used as negative control.

Incubation of the extract cells with the substrate cells:

- After at least 1 hour substrate slides were removed from the lysis solution.
- Slides were washed 3 times for 5 minutes with cold buffer F.
- Slides were placed on the metal units, covered with a silicon gasket with 12 holes and a top plate with corresponding holes. The parts are fixed with metal clamps to hold them together (see in figure 9).

- 30µl of the extracts, the positive and negative controls were pipetted onto the gels in the wells formed by the gasket. This was done on ice to prevent premature enzyme activity of the extract (pipetting scheme see figure 10).
- Slides were covered with a silicon seal and metal units were placed in a humidity chamber (plastic box filled with approx. 1cm of water at the bottom; metal units were ranged above without getting wet). Box was brought to a temperature of approx. 37°C before use.
- Slides were incubated at 37°C for exactly 30 minutes.
- In the meantime the electrophoresis was prepared.

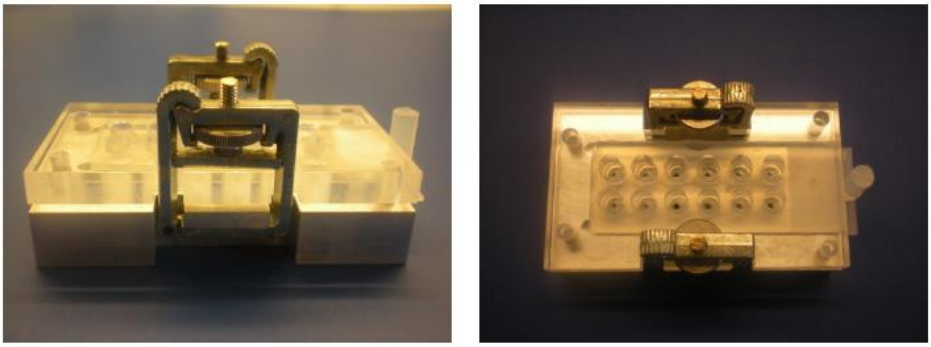

Figure 9. Slides placed, fixed and covered on the 12-Gel Comet assay metal units (Azqueta, Langie et al. 2013).

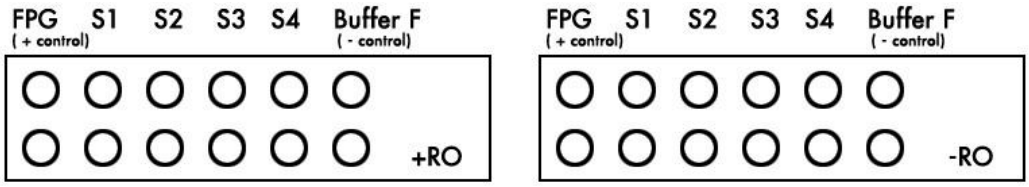

Figure 10. Pipette scheme for extracts, positive and negative controls.

Alkaline treatment and electrophoresis:

After the incubation time, metal units were removed from the incubator and immediately placed on ice for stopping the reaction time. Slides were carefully removed from the metal units to avoid that the glass slides get broken.

- Slides were placed in the electrophoresis tank (each row must be completed and therefore we used blank but pre-coated slides to fill the gaps).
- The cold electrophoresis puffer (2,000ml) was carefully filled in the electrophoresis tank. All slides must be covered with the solution.
- Slides were incubated for exactly 40 minutes with the electrophoresis solution (unwinding phase).
- Afterwards, electrophoresis was started with following settings: 25V, approx. 300mA, max. Watt, 30 minutes.

Neutralisation and washing steps:

When electrophoresis was done, slides were removed from the tank and placed, back to back, in a cuvette.

- Slides were neutralized by washing them
  - for 5 minutes with cold PBS,
  - for 5 minutes with bidestilled water,
  - for 15 minutes with 70% alcohol and
  - for 15 minutes with alcohol absolute.
- For drying the slides they were kept on a save place over night.
- The next day they were collected in a box and stored at 4°C.

Staining and image analyses:

- 30µl of the cooled dye GelRed was used for staining 6 gels.
- Stained cells were covered with a 22x22 cm cover slip.
- The cells were counted with a fluorescence microscope and computer-assisted image analysis software (Comet 5.5).
- After staining and counting the first 6 gels on the slide, the next 6 gels were stained and counted.
- From each study sample 100 cells were counted (2 gels of each 50 cells).

Calculating the repair rates:

The incision activity of the repair enzymes were expressed in tail intensity (TI % DNA in tail). We received following values of TI % DNA in tail:

- Background control: negative control (buffer F) incubated with non-exposed substrate shows the background DNA breaks (TI RO-/buffer).
- Treatment control: negative control (buffer F) incubated with exposed substrate shows the background DNA breaks and the breaks induced by the photosensitiser (TI RO+/buffer).
- Specifity control: extracts incubated with non-exposed substrate shows the background DNA breaks and non-specific breaks present in the extract (TI RO-/extract).
- The extract incubated with exposed substrate shows the background DNA breaks, induced breaks, non-specific breaks and the most interesting the specific extract incision (TI RO+/extract).

The background control value (TI RO-/buffer) must be subtracted from the other 3 values to receive the net breaks (net TI).

Calculation for the **repair related DNA incision** as follows:

Repair related DNA incision = net TI of (RO+/extract) – net TI of (RO-/extract) – net TI (RO+/buffer) (Azqueta, Langie et al. 2013).

3.2.5 Protein determination

The determination of the protein concentration in the extracts was done retrospectively (after the *in vitro* Comet repair assay) with the Bradford assay.

Seven standard concentrations with BSA in the range between 125–2,000µg/ml were prepared. Therefore, BSA was diluted with the same buffers applied in the Comet repair assay (mixture of buffer A and buffer F, 1:4). As blank sample we used the buffer mixture. A standard curve was calculated to determine the extract concentrations.

- 20µl of each extract was pipetted in small test tubes.
- 1ml of the Bradford dye (room temperature) was added to the extracts and thoroughly mixed.
- Tubes were incubated in the dark for 10 minutes.
- In the meantime, the spectrophotometer was adjusted to 595nm and set to zero with the blank sample.
- After 10 minutes, extracts were quickly transferred to 2.5ml micro cuvettes and inserted in the spectrophotometer and the absorbance value was read off.
- The protein concentrations of the extracts were calculated by linear regression (r^2 = 0.985 for the standard curve).

The repair related DNA incision, also called repair capacity (% DNA in tail) was expressed per 1mg protein (% DNA in tail/mg protein).

3.3 Statistical Analysis

Statistical analysis was performed using the software IBM SPSS Statistics 22.0. The data were group-wise proved for normal distribution by a 1-sample Kolmogorow-Smirnow test (KS-test).

As our data do not follow a normal distribution, further analysis was carried out using non-parametric tests. Statistical outliers were identified and viewed by boxplot graphics.

Median values between two groups were compared by using the non-parametric Mann-Whitney-U-test for independent samples. For more than two groups, the Kruskal-Wallis-test (K-independent samples) was used. If a significant distribution was observed by the Kruskal-Wallis-test, we performed a post-hoc analysis for non-parametric data in order to find significant differences between certain groups.

For testing bivariate correlations, we used the Spearman´s correlation test (non-parametric data).

The significant differences were marked with *. The significance levels $p<0.05$ marked with *, $p<0.01$ with **, and a highly significant level $p<0.001$ marked with ***. Nearly all observed results were expressed as mean value ± standard deviation (SD).

The UCB concentrations (µM) were divided into two groups (GS vs. control) and different test parameters, predominantly DNA repair capacity, were tested in the age- and gender-matched groups: in the GS, with a UCB concentration ≥17.1µM and in the control group (C), with a UCB concentration <17.1µM.

Furthermore, test variables were proven in UCB concentrations < and ≥10µM and in UCB (µM) quartiles. Moreover, all statistical performances were done after stratifying data for gender and age groups (<35 and ≥35 years).

4 RESULTS AND DISCUSSION

4.1 DNA Repair Capacity

We hypothesized, that GS subjects have a higher DNA repair capacity (% DNA in tail/mg protein) due to a postulated decreased risk for oxidatively-influenced chronic diseases for subjects with GS (Temme, Zhang et al. 2001, Lin, O'Donnell et al. 2006, Vitek, Novotny et al. 2006, Horsfall, Rait et al. 2011), and due to a suggested anti-genotoxic and anti-oxidative potential of bilirubin. The purpose of this work was to examine the DNA repair capacity of GS subjects and compare it with an appropriate gender- and age-matched control (C) group. Higher activity of human repair enzymes might be associated with a reduced risk for oxidatively-changed DNA and for developing oxidatively-influenced diseases.

Descriptive statistic:

	Men	**Women**	**Total study population**
Gilbert´s syndrome (n)	41	20	61
Control (n)	40	20	60
Total study population (n)	81	40	121

Table 3. Descriptive statistic of the total study population

Median	**GS**	**Control**	**Men**	**Women**
Age (years) ± SD	37 ± 14	37 ± 14	36 ± 14	41 ± 14
Repair capacity (% DNA in Tail/mg protein) ± SD	12.2 ± 4.1	12.4 ± 5.5	12.6 ± 5.1	11.6 ± 4.3
UCB (µmol) ± SD	32.9 ± 9.9	9.2 ± 3.4	22.4 ± 12.9	18.7 ± 11.7

Table 4. Median values of age, repair capacity and UCB in different groups

4.1.1 Repair capacity in Gilbert´s syndrome and control subjects

Within the repair capacity, one outlier (male, control group) was omitted (58.18% vs. 12.28% DNA in tail/mg protein) from the statistical analysis.

Unconjugated serum bilirubin (UCB) has been divided into two groups; GS and C. GS is characterized as mild hyperbilirubinemia with a UCB concentration of ≥17.1µM. Subjects with <17.1µM UCB were defined as the C group.

	Phenotype	**N**	**Median +/- SD (% DNA in Tail/mg protein)**	**p**
Repair capacity (% DNA in Tail/mg protein)	GS	61	12.2 ± 4.1	n.s.
	C	59	12.4 ± 5.5	

Table 5. Repair capacity in GS and C group (total study population)

The conducted analysis cannot confirm our hypothesis. Our results indicated no significant difference regarding the repair capacity between the two investigated groups.

Chang et al. observed a lower repair capacity in subjects with the homozygote UGT1A1*28 genotype (GS) compared to the wild-type genotype (Chang, Chen et al. 2010).

Control and GS subjects in this study are generally considered as healthy subjects and might have low levels of DNA damage. Additionally, in the *in vitro* comet repair assay, the used substrate exhibited a comparatively low density of lesions and simulated real life conditions (Godschalk, Ersson et al. 2013).

A study pointed out, that DNA repair enzymes are induced by DNA damage but the repair mechanisms evolve their maximal intensity of repair only after maximal induction of DNA damage (Langie, Koppen et al. 2015).

Low levels of DNA damage might not even trigger the repair enzymes and therefore, the repair capacity was not increased at all.

Results after gender stratification

The repair capacity of enzymes to repair oxidative DNA damage is observed in GS and C subjects after stratification in male and female data.

			N	Median ± SD (% DNA in Tail/mg protein)	p
Repair capacity (% DNA in Tail/mg protein)	males	GS	41	12.2 ± 4.1	n.s.
		C	39	13.1 ± 5.9	
	females	GS	20	12.0 ± 4.3	n.s.
		C	20	11.2 ± 4.3	

Table 6. Repair capacity in GS and C group regarding sex (total study population)

The repair capacity of men and women did not differ significantly between the GS and the C group. Within the group of men, the control subjects had a 6.6% higher repair capacity compared to the GS subjects. Opposite results were found in the women group: the GS group showed an 8% higher repair capacity in comparison to the control group. This data might suggest that bilirubin concentration influences the repair capacity in a gender-dependent manner, although differences were not significant.

The mean repair capacity was 12.28 ± 4.81 for the total study population. Results summarized in the table below showed the influence of gender on the repair capacity in the total study population.

		N	Median +/- SD (% DNA in Tail/mg protein)	p
Repair capacity (% DNA in Tail/mg protein)	males	80	12.6 ± 5.1	n.s.
	females	40	11.6 ±4.3	

Table 7. Influence of gender on the repair capacity (total study population)

A significant difference between males and females could not be demonstrated, although male subjects did show an 8% higher repair activity than females.

A study pointed out that male subjects have greater DNA damage than females (Loft, Vistisen et al. 1992), which might induce a higher activity of repair enzymes in men.

A study performed in 340 Norwegians demonstrated that females exhibit higher levels of single-strand breaks ($p=0.035$) and a lower level of BER ($p=0.027$) and NER ($p=0.004$) activity when compared to male subjects. They concluded that the gender aspect significantly modulates the levels of DNA damage and repair (Slyskova, Lorenzo et al. 2014).

Garm et al. observed 216 subjects, 68% women and 32% men aged between 40-77 years with regard to their SSB- and DSB repair capacity. They demonstrated no gender differences between the analyzed repair parameters, SSB- and DSB-repair (Garm, Moreno-Villanueva et al. 2013).

A study measured the activity of the BER enzyme 8-oxoguanine glycosylase (OGG1) with the OGG assay. They analyzed 120 subjects with an age of 55 years and younger and observed that females show a decreased OGG1 activity in comparison to males. Furthermore, it was hypothesized that the activity of DNA ligation and polymerization in the repair pathways, BER and NER, was lower in white women than in white men (Paz-Elizur, Elinger et al. 2007).

Results after age stratification

The study population was divided into two age groups (<35 and ≥35 years) and repair capacity was analyzed in GS and control groups for each age group.

			N	Median ± SD (% DNA in Tail/mg protein)	p
Repair capacity (% DNA in Tail/mg protein)	<35 years	GS	34	12.0 ± 3.1	n.s.
		C	32	13.0 ± 6.0	
	≥35 years	GS	27	12.4 ± 5.2	n.s.
		C	27	11.8 ± 4.8	

Table 8. Repair capacity in GS and C group regarding age (total study population)

For both age groups, no significant difference of the repair capacity between GS and control was observed. Similar to the gender variation, there might be an age-specific impact of UCB concentrations on the repair activity.

The impact of age on the repair capacity in the whole study population is demonstrated in the table 9.

		N	Median +/- SD (% DNA tail)	p
Repair capacity (% DNA in Tail/mg protein)	<35 years	66	12.5 ± 4.70	n.s.
	≥35 years	54	12.1 ± 4.98	

Table 9. Influence of age on the repair capacity (total study population)

Overall, the repair capacity of the younger and the older age group did not show any difference at all.

4.1.2 Repair capacity and UCB concentration <10µM and ≥10µM

Evidence from the literature demonstrated a protective effect of UCB from 10µM and upwards. So the present data were also calculated for this threshold.

		N	Median ± SD (% DNA in Tail/mg protein)	p
Repair capacity (% DNA in Tail/mg protein)	<10µM UCB	37	11.1 ± 4.9	**0.028***
	≥10µM UCB	83	**12.8 ± 4.7**	

Table 10. Repair capacity analyzed for <10µM UCB and ≥10µM UCB concentration (total study population)

The result showed that the repair capacity was significantly higher (16%) in subjects with a UCB concentration greater than 10µM. This result suggests a positive effect of UCB on the repair capacity already seen from lower concentrations.

A protective effect of a total bilirubin level greater than 10µmol/L was also suggested in association with diabetes. In nearly 16,000 subjects, it could be demonstrated that subjects with a bilirubin level ≥10µmol/L had a 20% lower risk for developing diabetes when compared to subjects with a bilirubin level <10µmol/L (Cheriyath, Gorrepati et al. 2010).

The 10µM/L threshold is chosen since the average UCB concentration of the general population represents this value (10µM/L) (Bulmer, Blanchfield et al. 2008).

Gender specific results for the 10µM UCB threshold

The table below provides insights into the gender-dependent effects regarding the UCB concentration <10µM and ≥10µM.

			N	Median ± SD (% DNA in Tail/mg protein)	p
Repair capacity (% DNA in Tail/mg protein)	male	<10µM UCB	22	11.1 ± 5.6	**0.026***
		≥10µM UCB	58	**13.2 ± 4.8**	
	female	<10µM UCB	15	11.1 ± 3.8	n.s.
		≥10µM UCB	25	11.9 ± 4.6	

Table 11. Repair capacity analyzed for <10µM and ≥10µM UCB, regarding sex (total study population)

A significantly higher repair capacity was found in men with a UCB level above 10µM. Men (≥10µM UCB) showed a nearly 20% higher repair capacity compared to men with a lower UCB concentration.

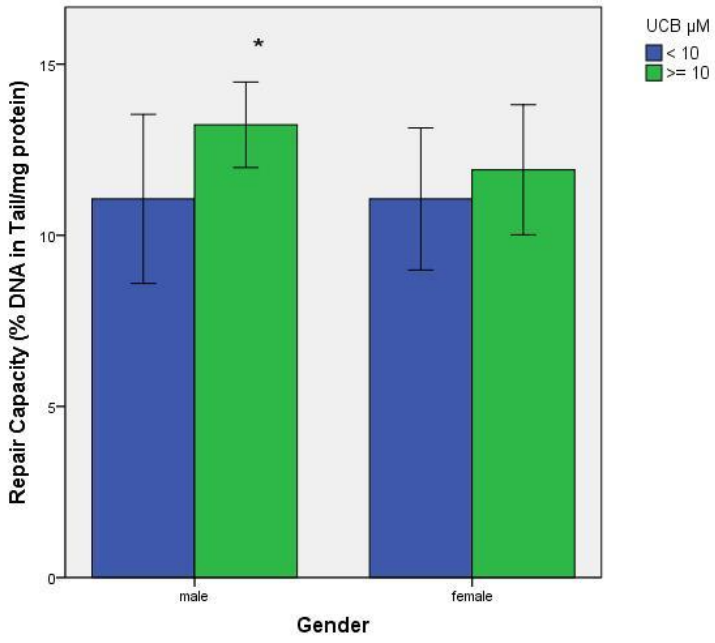

Figure 11. Repair capacity analyzed for <10µM and ≥10µM UCB, regarding sex (total study population)

A Korean study included 603 men and 402 women and presented that women with a bilirubin level <10µM in comparison to women with a higher level had a 5-fold increased risk for leukoaraiosis, which is a risk factor for cardiovascular diseases (Park, Shim et al. 2012).

Supporting evidence for protective effects of UCB ≥10 µM was provided by a 10 year follow-up study (men n=5,460, women n=4,843). It has been shown that men with normal high serum bilirubin levels (≥10.3µM) in contrast to normal low levels (<3.4µM) have a lower relative risk (RR) for all-cause mortality (RR=0.73) and cancer mortality (RR=0.42) (Temme, Zhang et al. 2001).

Age specific results for the 10µM UCB threshold

The influence of age on the repair capacity at UCB levels <10µM and ≥10µM are summarized in the table below.

			N	Median ± SD (% DNA in Tail/mg protein	p
Repair capacity (% DNA in Tail/mg protein)	**<35 years**	<10µM UCB	16	11.0 ± 5.2	0.054
		≥10µM UCB	50	12.9 ± 4.5	
	≥35 years	<10µM UCB	21	11.1 ± 4.7	n.s.
		≥10µM UCB	33	12.7 ± 5.1	

Table 12. Repair capacity analyzed for <10µM UCB and ≥10µM UCB, regarding age (total study population)

The association between a UCB threshold level of ≥10µM and the repair capacity was more obvious in the younger study group than in the older study group and was almost significant in the younger group.

When we performed the analysis for men, statistical significance could not be achieved. This might be based on the lower number of subjects in the subgroups.

Men			N	Median ± SD (% DNA in Tail/mg protein	p
Repair capacity (% DNA in Tail/mg protein)	**<35 years**	<10µM UCB	9	11.4 ± 6.5	n.s.
		≥10µM UCB	39	13.4 ± 4.6	
	≥35 years	<10µM UCB	13	10.8 ± 5.1	n.s.
		≥10µM UCB	19	12.9 ± 5.2	

Table 13. Repair capacity analyzed for <10µM and ≥10µM UCB in men, regarding age

Future investigations should focus on normal high bilirubin values, as they are affecting more people and might already have protective effects.

4.1.3 Repair capacity in UCB quartiles

To improve the understanding of the repair capacity at different UCB levels, we divided UCB concentrations in quartiles. The repair capacity was analyzed in the four UCB groups and the results are shown in table 14.

		N	Median ± SD (% DNA in Tail/mg protein)	p
Repair capacity (% DNA in Tail/mg protein)	1.4–8.9µM UCB	30	11.4 ± 4.8	n.s.
	9.0–17.9µM UCB	30	13.3 ± 5.9	
	18.0–31.9µM UCB	30	12.0 ± 4.2	
	32.0–53.2µM UCB	30	12.5 ± 4.2	

Table 14. Repair capacity analyzed in UCB quartiles (total study population)

No significant difference in the UCB quartiles regarding the repair capacity could be observed. The repair capacity reached its maximum at a UCB concentration of 9.0–17.9µM. The median values of the UCB quartiles were 6.4µM, 12.2µM, 25.1µM and 41.3µM UCB.

Gender and age specific results for the UCB quartiles

The influence of gender on the repair capacity was observed in the table below.

Women		N	Median ± SD (% DNA in Tail/mg protein)	p
Repair capacity (% DNA in Tail/mg protein)	3.2–8.1µM UCB	10	11.5 ± 3.0	n.s.
	8.2–15.8µM UCB	10	11.0 ± 5.6	
	15.9–30.6µM UCB	10	11.5 ± 4.7	
	30.7–45.5µM UCB	10	12.7 ± 4.0	

Table 15. Repair capacity analyzed in UCB quartiles, women

Results in women showed no significant difference in the repair capacity within the four UCB groups. The median values of the UCB quartiles were 5.8µM, 10.9µM, 24.3µM and 34.6µM UCB.

When observed in the two age groups (<35; ≥35 years), the repair capacity of younger women was significantly different (p=0.046) in the four UCB groups. The post-hoc analysis did not show a significant difference between the certain groups. The repair capacity of younger women was highest in the forth UCB quartile and lowest at the second UCB quartile (Fig. 12).

A statistically significant difference between the four UCB groups in the older women (≥35 years) could not be observed.

In the certain UCB quartiles no significantly different repair capacity between the two age groups (<35; ≥35 years) could be achieved which might be based on the lower number of subjects in the subgroups.

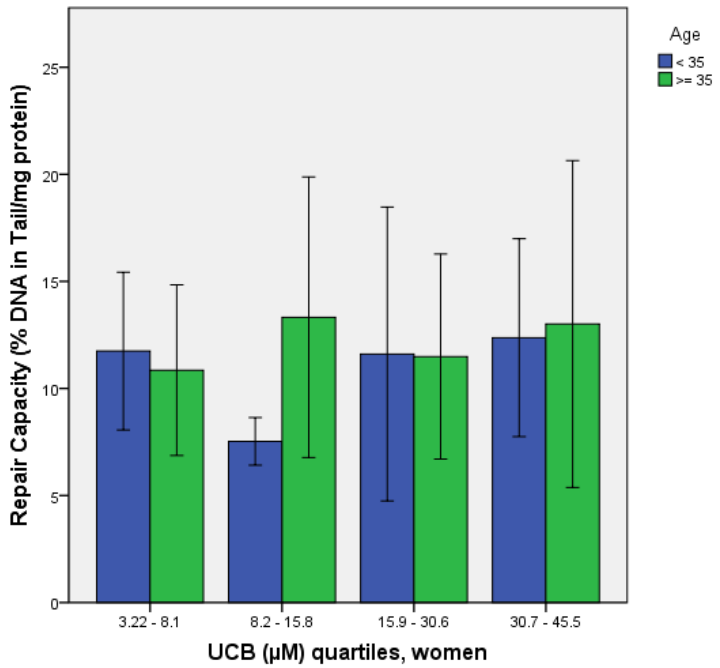

Figure 12. Repair capacity analyzed in UCB quartiles in women, stratified for age

Men showed a significance between the four UCB groups.

Men		N	Median ± SD (% DNA in Tail/mg protein)	p
Repair capacity (% DNA in Tail/mg protein)	1.7–9.1µM UCB	21	11.3 ± 5.6	**0.048***
	9.2–17.1µM UCB	18	**15.1 ± 5.8**	
	17.2–36.5µM UCB	21	11.6 ± 4.0	
	36.6–53.2µM UCB	20	12.9 ± 4.3	

Table 16. Repair capacity analyzed in UCB quartiles, men

A significant difference in men's repair capacity between the UCB quartiles could be shown. Again the post-hoc analysis did not show a significant difference between the various groups. Men´s repair activity was highest at a UCB concentration 9.2–17.1µM which also reflected the previous findings that men's repair capacity is significantly higher at a UCB concentration ≥10µM. The mean values of men´s UCB quartiles were 6.9µM, 12.7µM, 26.0µM and 44.3µM UCB.

Also in younger men (<35 years) a statistical significant difference (p=0.045) of the repair capacity in the four UCB quartiles could be achieved. No statistical significant difference was shown in the UCB quartiles of older men (≥35 years).

Similar to the findings in women, no significant difference in men´s repair capacity between the two age groups (<35; ≥35 years) could be determined in the different UCB quartiles which might be again due to the lower number of subjects in the subgroups.

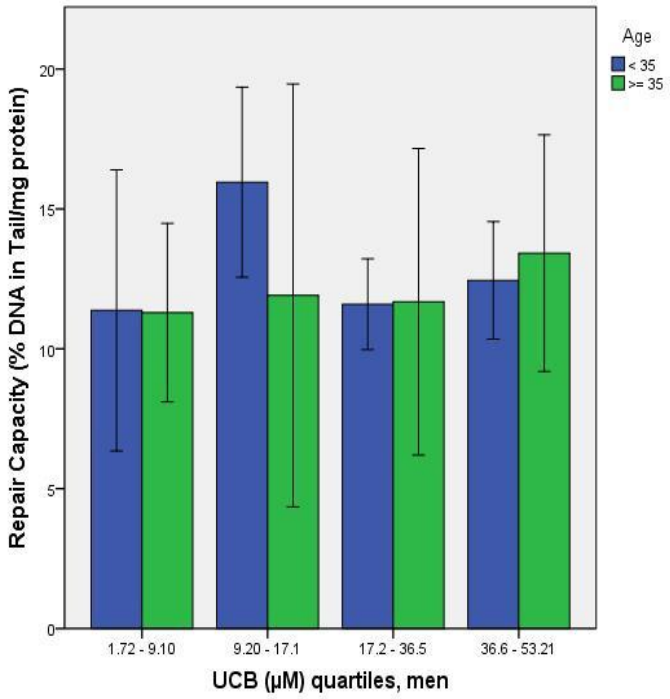

Figure 13. Repair capacity analyzed in UCB quartiles in men, stratified for age

Both men and women showed significant different repair capacity between the analyzed UCB quartiles in the age group younger than 35 years. In the 2nd quartile, younger men showed a much higher repair capacity (34%) than older men. For women, the result was opposite: In the 2nd quartile younger women showed a lower repair capacity (76%) than older women. Significant differences between the two age groups (<35; ≥35 years) in the certain UCB quartiles could not be achieved.

According to these results, it seems probable that different UCB levels have an impact on the repair capacity in the younger generation. Additionally, this seems

true for a UCB level that ranges from approximately 8–17 µM. Whether the UCB level is below or above this UCB area, it seems to have less effect on the repair capacity. It is interesting to note that this possibly “effective” UCB area leads to opposite results in younger men and women - young women in this UCB area have a much lower, and young men a much higher, repair capacity.

4.2 Correlations

4.2.1 Correlation between Repair capacity and UCB concentration

No correlation between repair capacity and UCB concentration was observed: for the whole study population, as well as for gender groups.

		Repair capacity (% DNA in Tail/mg protein)	**UCB (µM)**
Repair capacity (% DNA in Tail/mg protein)	r	1	0.106
	p	.	n.s.
UCB (µM)	r	0.106	1
	p	n.s.	.

Table 17. Correlation between repair capacity and UCB (total study population)

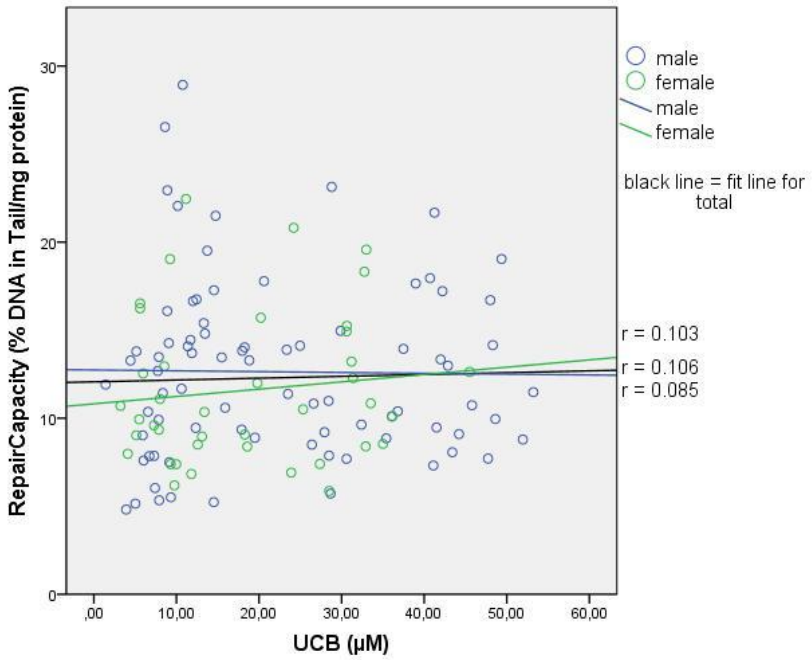

Figure 14. Correlation between repair capacity and UCB, stratified for gender

No

correlation could be shown between repair capacity and UCB levels, neither in the general population (table 17) nor in male (r=0.085) and female (r=0.103) subjects (Figure 14).

If observed in the two age groups (<35, ≥35 years), only the younger women showed a slightly positive correlation (r=0.201), but statistical significance could not be reached. In younger men and in the older age group, no correlation could be demonstrated.

In table 18, the correlation between repair capacity and the UCB level ≥10µM is shown.

		Repair capacity (% DNA in Tail/mg protein)	**UCB ≥10µM**
Repair capacity (% DNA in Tail/mg protein)	r	1	-.135
	p	.	0.223
UCB ≥10µM	r	-.135	1
	p	0.223	.

Table 18. Correlation between repair capacity and UCB ≥10µM (total study population)

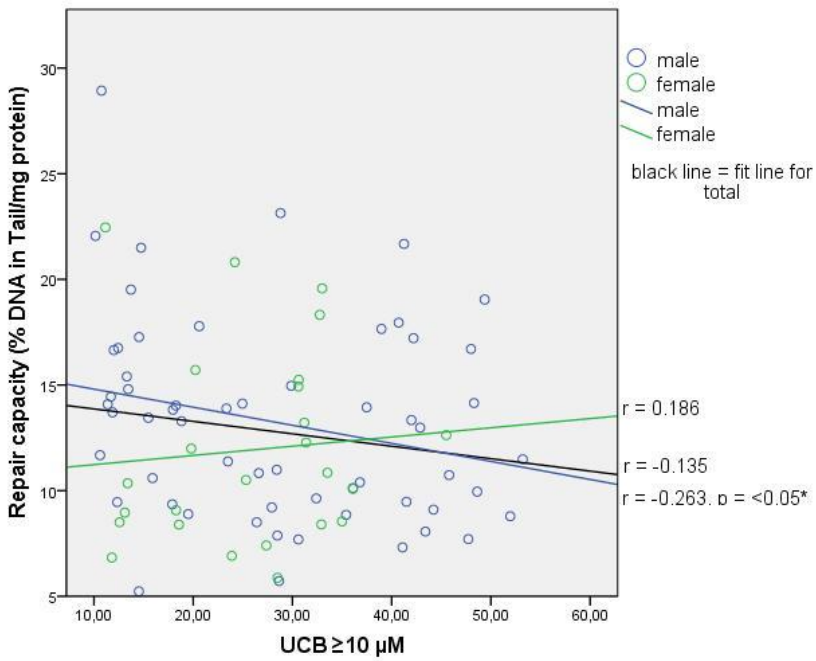

Figure 15. Correlation between repair capacity and UCB ≥10µM, distinguished between gender

For the total study population (black line), no correlation (r=-0.135) could be observed between the two parameters. Also, no correlation (r=0.186) could be shown for women.

In male subjects, a significant but weak negative correlation (r=-0.263; p=0.018) could be observed (Figure 14). As shown before, men had a significantly higher repair capacity at a UCB concentration higher than 10µM. As the above figure suggests, this repair capacity is declining when the UCB levels are raised further. This observation leads to the suggestion that the maximum repair capacity of men is around 10µM.

Furthermore, the younger study population (<35 years) was included in the correlation test between repair capacity and UCB concentration ≥10µM.

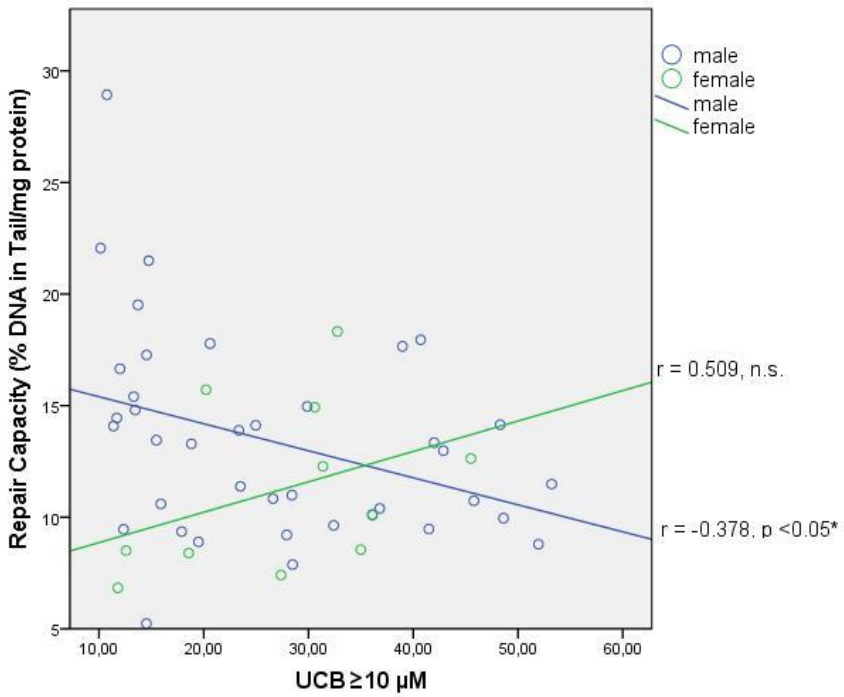

Figure 16. Correlation between repair capacity and UCB ≥10µM in the age group <35 years, stratified for gender

Within the younger men, a significant negative correlation (r=-0.378; p=0.018*) between UCB ≥10µM and repair capacity could be demonstrated. The younger female population showed a strong positive correlation (r=0.509), however statistical significance was lacking (Figure 15).

These findings support the previous suggestion that the UCB levels of younger men and women lead to opposite effects: In younger women the repair capacity is lowest in the UCB concentration range of 8.2–15.8µM and is then increased, while in younger men, the repair capacity is highest in the UCB range of 9.2-17.1µM, and then significantly decreases.

4.2.2 Correlation between Repair capacity and age

In the following, the relationship between age and repair capacity is discussed.

		Repair capacity (% DNA in Tail/mg protein)	Age (20-77 years)
Repair capacity (% DNA in Tail/mg protein)	r	1	-0.077
	p	.	0.402
Age (20-77years)	r	-0.077	1
	p	0.402	.

Table 19. Correlation between repair capacity and age (total study population)

No correlation could be found between age and repair capacity in the entire study population (black line in the figure below).

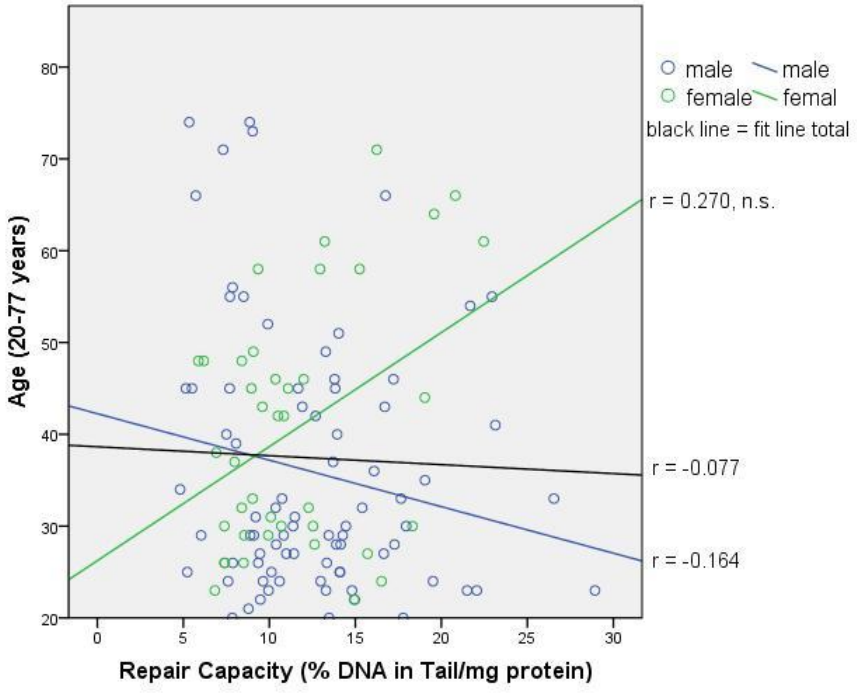

Figure 17. Correlation between repair capacity and age, stratified for gender

In women, a positive correlation (r=0.270) could be observed, although statistical significance (p=0.092) was lacking. No correlation (r=-0.164) could be observed in male subjects.

Within the younger (<35 years) study group, no correlation between the two parameters could be observed.

A weak negative correlation (r=-0.277, p=0.124) could be demonstrated for the ≥35 year old men.

The correlation between women´s repair capacity and age became stronger when only the ≥35 year old women were considered. A significantly strong positive correlation (r=0.549, p=0.008**) was observed.

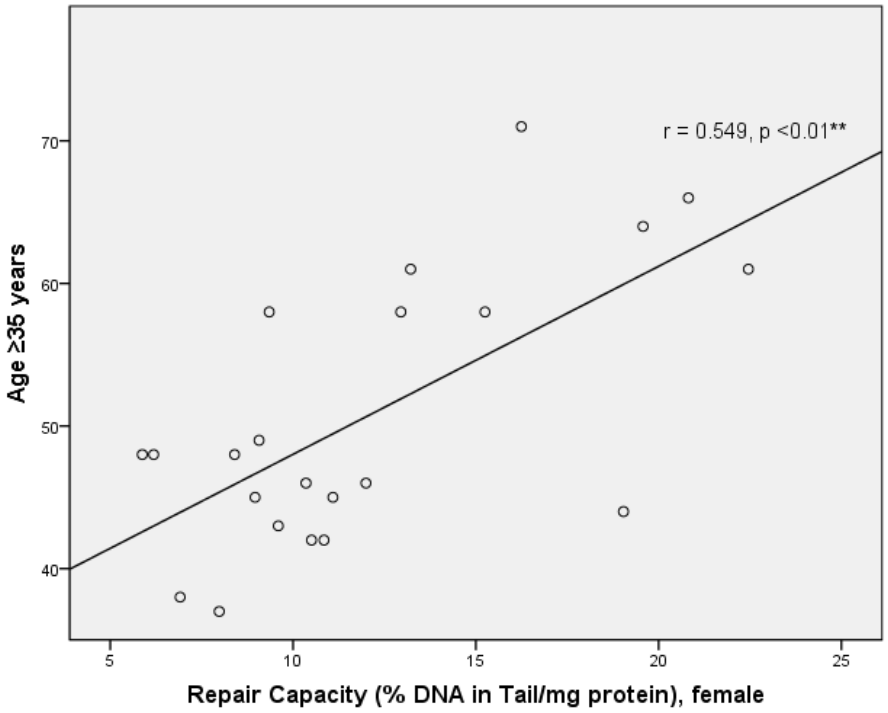

Figure 18. Correlation between repair capacity and ≥35 year aged females

The above finding suggests, that women´s repair capacity increases with age.

A group of investigators examined the impact of age, sex, and ethnicity on the repair activity of single-strand breaks (SSBs) in a human population. They pointed out that changes in the repair capacity with age varied inconsistently with gender and ethnicity. Regarding ethnicity, they had shown that the rate of the fast component of SSBs repair in white women is faster with age, whereas in African-American women, the rate is slower with age (Trzeciak, Barnes et al. 2008).

Their findings in terms of age and gender are in support of our results – the repair capacity in women increases with the age whereas the repair capacity in men decreases with the ageing process.

Humphreys et al. conducted a study and showed that the oldest age group (75-82 years) had the greatest level of anti-oxidative defense and the greatest resistance to oxidative damage in comparison to the age groups 63–70 years and 20–35 years. Furthermore, they demonstrated that the repair activity increases with age over all age groups for both genders. They suggest that increased DNA repair activity in older subjects results in a response to an elevated release of ROS due to senescent mitochondria (Humphreys, Martin et al. 2007).

A study performed in 375 subjects underlines this finding. They observed a significant increase in the DNA repair activity with age for both men and women (Dusinska, Dzupinkova et al. 2006).

4.2.3 Correlation between UCB and BMI

The relationship between UCB concentrations and BMI was examined in the following observations.

		UCB (µM)	**BMI**
UCB (µM)	r	1	**-0.251**
	p	.	**0.006****
BMI (kg/m^2)	r	-0.251	1
	p	0.006**	.

Table 20. Correlation between UCB and BMI (total study population)

A significant, negative correlation was observed between the two parameters. This means that a higher UCB concentration is associated with a lower BMI in the whole study population.

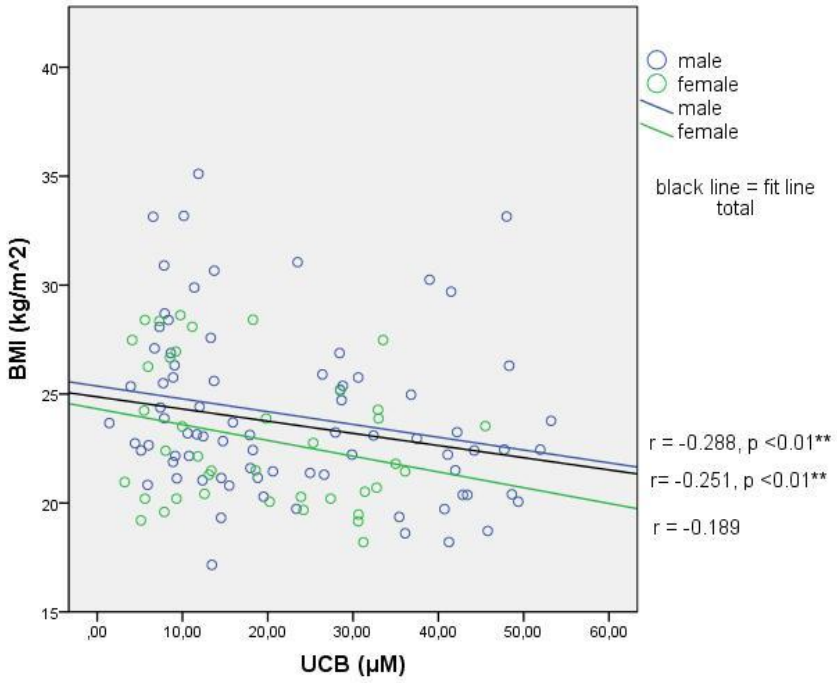

Figure 19. Correlation between UCB and BMI, stratified for gender

When considering the gender factor, a significant, weak negative correlation (r=-0.288, p<0.010**) could be observed for male subjects. Furthermore, the negative correlation in men became stronger when only younger men (<35 years) were analyzed (r=-0.355; p=0.019*). No relationship could be demonstrated for women (r=-0.189).

Similar findings were provided by a study performed by Wallner et al. who demonstrated higher UCB µM concentration in the group with lower BMI (Wallner, Antl et al. 2013).

It is well known that losing weight decreases cardiovascular risk factors. Therefore, it is noteworthy that each percent reduction in weight loss is related to a linear increase in the serum bilirubin concentration (Andersson, Weeke et al. 2009).

4.2.4 Correlation between Repair capacity and BMI

Due to the significant correlation found between the UCB concentration and the BMI, we also explored the relationship between the repair capacity and BMI.

		Repair capacity (% DNA in Tail/mg protein)	BMI (kg/m^2)
Repair capacity (% DNA in Tail/mg protein)	r	1	0.028
	p	.	0.764
BMI (kg/m^2)	r	0.028	1
	p	0.764	.

Table 21. Correlation between repair capacity and BMI (total study population)

As table 21 indicates, there was no correlation between repair capacity and BMI in the entire study population. Also, when dividing the study population into men (r=0.042) and women (r=-0.085) no correlation could be found.

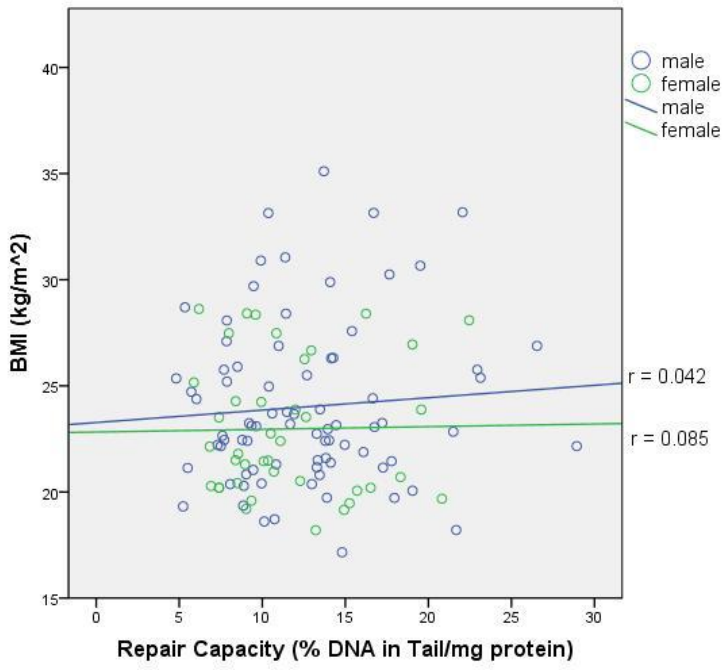

Figure 20. Correlation between repair capacity and BMI, stratified for gender

Thus, we can conclude that an individual's BMI is not linked to the repair capacity in the present study.

4.3 Linear Regression

A great variety of parameters might influence the repair capacity and were included in the linear regression model to uncover the most descriptive variables for the repair capacity. The included variables in the model were: Age (years), BMI (kg/m^2), albumin (mg/ml), UCB (µM), heme (µM), total cholesterol (mg/dl), HDL cholesterol (mg/dl), LDL cholesterol (mg/dl), triglyceride (mg/dl), C-peptide (ng/ml), IL-6, IL-1b, TNF, C-reactive protein (CRP, mg/dl), sum of activity score, endurance exercise, and resistance exercise.

Tested subjects	Predictors	Adjusted R^2	p-value
Total study population (87)	Albumin	0.035	0.045*
<35 years (50)	Albumin	0.127	0.002**
	IL 6	0.196	0.027*
Men (54)	Albumin	0.095	0.002**
	IL 6	0.184	0.012*
<35 years (35)	Albumin	0.105	0.030*
>35 years (18)	Age	0.179	0.040*
Women (32)	Total cholesterol	0.208	0.009**
	C-reactive protein	0.284	0.047*
<35 years (14)	HDL cholesterol	0.275	0.026*
>35 years (17)	Age	0.407	0.000***
	C-reactive protein	0.640	0.004**

Table 22. Linear regression model to predict repair capacity

The table above summarizes the stepwise linear regression model for the repair capacity.

In the total study population, albumin explained 3.5% of the repair capacity. That is not surprising since albumin is responsible for UCB transport in the bloods stream

Together with IL 6, albumin explained nearly 20% of the repair capacity in the younger study group while no predictor variable for the older study group could be found.

Men´s repair capacity was described by 18.4% through albumin together with IL 6. Albumin with 10.5% also had the greatest impact on the repair capacity in younger men. In men older than 35 years, repair capacity was influenced the most (18%) by age.

The repair capacity of women was described by different predictors. Total cholesterol and CRP explained 28.4% of women´s repair capacity. The most influencing factor in younger females was HDL cholesterol with 27.5%.

As described in older men, the same was true for older women; age explained 40.7% of the repair capacity of older women and summed up to a total impact of 64% together with CRP. This lead to the assumption, that the repair capacity of older women is increased (as demonstrated before) possible due to higher levels of oxidative stress caused by elevated levels of chronic inflammation. C-reactive protein is associated with chronic inflammation and plays an important role as a biomarker of oxidative stress-related diseases, particularly in elderly people.

A study performed by Trzeciak et al. suggested that sex, red blood reduced glutathione concentration, heme intermediates and the serum concentration of high-sensitivity CRP have an impact on the extent of SSBs, Endo III labile sites and the repair capacity of SSBs (Trzeciak, Mohanty et al. 2012).

5 CONCLUSION

The purpose of this study was to examine the DNA repair capacity in subjects with GS (≥17.1µM UCB) compared with subject allocated to the control group (<17.1µM UCB). Moderately elevated UCB levels (GS) are considered to have anti-oxidative and anti-mutagenic effects. We analyzed the link between different UCB serum concentrations and the DNA repair capacity.

A decreased risk for CVDs and cancer is pronounced for subjects with GS. This gives rise to our hypothesis that moderately elevated UCB levels may have an impact on the activity of repair enzymes and thereby repair oxidative DNA damage more efficiently, which in turn protects against oxidatively-induced chronic diseases and genetic instability.

We could not confirm our hypothesis that GS subjects have a higher DNA repair capacity compared to the control subjects. Nevertheless, the study leads to interesting results. The repair capacity was significantly higher when UCB concentrations reached a threshold above ≥10µM. This significant difference was clearly demonstrated in men but not in women and tended to be true only for the younger population in the study.

The repair capacity of younger men was highest at a UCB concentration between 9.2–17.1µM (median value 12.7µM) and showed a significantly negative correlation with increasing UCB concentrations. This result indicates a decreasing repair capacity in men with increasing UCB concentration.

The repair capacity of younger women was lowest at a UCB concentration between 8.2–15.8µM (median value 10.9µM) and exhibited an insignificantly strong positive correlation with increasing UCB levels, suggesting that the repair capacity in women increases with increasing UCB concentration.

The repair capacity of older women significantly increases at the age of ≥35 years. The opposite result was achieved for older men (negative correlation) without reaching statistical significance.

Furthermore, a significant negative correlation between UCB µM levels and BMI could be demonstrated for male subjects. This correlation was even stronger when only younger men (<35 years) were considered. In the GS group, no correlation could be observed between the two parameters, neither in male nor in female subjects. However, men with GS had a significantly lower BMI in comparison to the control subjects.

There is a missing link between protein concentration and repair capacity. Therefore, it is recommended for the extracts (human study samples) that a constant cell or protein concentration is adjusted before the incubation step with the cell substrates. This is the only way to ensure that all damaged cells are treated with the same amount of repair enzymes in the extract (Azqueta, Langie et al. 2013). The recommended protein concentration for lymphocytes is 2mg/ml (Langie, Knaapen et al. 2006). The optimal protein concentration of the extract should be analyzed in each laboratory. The laboratory differences in the incubation of the extract cells with the substrate cells might be the main cause for inter-laboratory variation when analyzing the repair capacity with the *in vitro* comet repair method (Godschalk, Ersson et al. 2013). The protein concentration of our extracts ranged between 0.32–2.88mg/ml (median value 1.39±0.51 SD mg/ml).

6 SUMMARY

Literature from the last decade indicates that moderately elevated serum bilirubin concentrations are associated with a reduced risk for chronic diseases, particularly, for CVDs and more recently, for cancer (lung and colorectal cancer) in humans. The underlying mechanism behind bilirubin´s protective effect in disease prevention is not fully understood but it´s anti-oxidative and anti-mutagenic properties are one major point of discussion.

In our human case-control study we hypothesized that GS subjects have a higher capability to repair oxidative DNA damage more efficiently than control subjects. This would add another mechanism to explain why subjects with GS are better protected against chronic diseases. One hundred twenty subjects were enrolled in the study and were allocated according to their UCB concentrations in gender- and age-matched groups. Peripheral Blood Mononuclear Cells (PBMCs) of the GS group (≥17.1µM UCB, n=60) and of the control group (<17.1µM UCB, n=60) were analyzed for their DNA repair capacity with the "*in vitro* Comet repair assay". The *in vitro* Comet repair assay is a modified version of the Single Cell Gel electrophoresis (SCGE/Comet Assay) with one additional step: lesion-containing nucleoids are digested with lesion-specific endonucleases to detect oxidized bases. The investigated repair pathway within this assay is the base excision repair pathway (BER) which primarily detects 8-oxo- 2´deosyguanosine lesions (8-oxodG).

We could not confirm our hypothesis that a more efficient DNA repair capacity is one major mechanism in protection of GS subjects. However, an age- and gender-dependent impact of elevated bilirubin concentration on the repair capacity can be suggested. Nevertheless we could demonstrate that slightly elevated serum bilirubin levels (≥10µM UCB) is linked to significantly increased repair activity. In the literature an association between slightly elevated serum bilirubin concentration (≥10µM UCB) and a reduced prevalence of CVDs and cancer is reported.

Furthermore, contrary results for men and women were achieved with regard to the relationship between repair capacity and age. While the repair capacity of women increases, the repair capacity of men decreases in the ageing process.

It is suggested that an individual´s repair capacity is influenced by a variety of factors. We conclude that age, sex, and the antioxidant status have an impact on an individual´s repair capacity. We can also conclude that the investigated study groups were generally considered as healthy and thereby have low levels of DNA damage, which might not even trigger the repair enzymes. Further studies are needed to improve the understanding behind the complexity of DNA repair pathways. Additionally, future investigations should be done for a UCB concentration from 10µM on as they are affecting more people and might already have protective effects.

7 ZUSAMMENFASSUNG

Studien der letzten zehn Jahre lassen auf einen Zusammenhang zwischen einer moderat erhöhten Serumbilirubin-Konzentration und einem verminderten Risiko für chronische Erkrankungen (Herz-Kreislauf Erkrankungen, Krebs) beim Menschen schließen. Die zugrundeliegenden Mechanismen sind nicht vollständig bekannt, jedoch liefern die antioxidativen und antimutagenen Eigenschaften von Bilirubin wichtige Erklärungsansätze und sind Gegenstand aktueller wissenschaftlicher Diskussionen.

In der durchgeführten Fall-Kontroll Studie wurde die Hypothese aufgestellt, dass Personen mit Gilbert Syndrome (GS) im Vergleich zur Kontrollgruppe eine höhere DNA-Reparaturkapazität aufweisen und somit oxidative DNA Schäden effizienter reparieren können. Eine Bestätigung dieser Annahme würde einen weiteren schützenden Mechanismus von Bilirubin belegen und eine weitere Erklärung des geringeren Risikos von Personen mit GS in Bezug auf chronische Erkrankungen liefern.

Einhundert zwanzig Studienteilnehmer wurden basierend auf ihrem unkonjugierten Bilirubinspiegel (UCB, µM) in zwei Gruppen aufgeteilt. Die GS Gruppe (≥17,1µM UCB, n=60) wurde mit der nach Alter und Geschlecht gematchten Kontrollgruppe (<17,1µM UCB, n=60) hinsichtlich ihrer DNA-Reparaturkapazität verglichen. Hierfür wurde mit der Methode „*in vitro* Comet Repair assay“ die Aktivität der Reparaturenzyme, in den aus Vollblut isolierten Lymphozyten, analysiert. Diese Methode ist eine um einen Schritt erweiterte Version der Einzelzellgelelektrophorese (SCGE, Comet Assay): Die läsion-haltigen Nukleotide werden mit einer spezifischen Endonuclease inkubiert, welche die oxidativ-geschädigte DNA Base identifiziert. Mit der Methode „*in vitro* Comet Repair assay“ wird der Mechanismus der Basenexzisionsreparatur analysiert der die oxidative Basenmodifikation, 8-Oxo-2´-desoxyguanosin (8-OxodG) erkennt und repariert.

Die Annahme, dass Personen mit GS eine höhere DNA-Reparaturkapazität aufweisen, wurde nicht bestätigt. Es zeigte sich jedoch, dass eine erhöhte UCB Konzentration (≥17,1µM) einen alters- und geschlechtsspezifischen Einfluss auf die Reparaturkapazität hat. Interessanterweise führte bereits eine leicht erhöhte UCB Konzentration von ≥10µM zu einem Zusammenhang mit einer signifikant höheren DNA-Reparaturkapazität. In bisherigen Studien wurde bereits eine leicht erhöhte UCB Konzentration (≥10µM) mit einem verminderten Risiko für kardiovaskulären Erkrankungen und Krebs in Verbindung gebracht. Auch unabhängig von der UCB-Konzentration kann ein geschlechtsspezifischer Zusammenhang zwischen Alter und Reparaturkapazität angenommen werden: Die DNA-Reparaturkapazität der Frauen steigt mit zunehmenden Alter während sie bei den Männer mit zunehmenden Alter sinkt.

Die Ergebnisse dieser Studie lassen darauf schließen, dass Bilirubin, Geschlecht und Alter die DNA-Reparaturkapazität beeinflussen. Weiters wird vermutet, dass die untersuchte, generell gesunde, Studienpopulation einen geringen basalen DNA Schaden aufweist und somit keine Reparaturenzyme induziert wurden. Weitere Studien sind nötig um die Komplexität der Reparaturmechanismen zu verstehen. Ein besonderer Fokus sollte dabei auf den Zusammenhang zwischen einer leicht erhöhten UCB-Konzentration (≥10µM) und einem geringeren Krankheitsrisiko gelegt werden, da eine deutlich größere Bevölkerungsgruppe, als nur Personen mit GS, davon betroffen ist.

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9 CURRICULUM VITAE

Persönliche Daten

Name: Petra Hinterleitner, BSc
Staatsangehörigkeit: Österreich
Geburtsdatum: 14.11.1984

Ausbildung

Seit 02/2013	Masterstudium Ernährungswissenschaften, Molekulare Ernährung, Universität Wien Masterthesis: „Evaluation of the DNA Repair capacity in subjects with Gilbert´s Syndrome".
10/2008–01/2013	Bakkalaureatsstudium Ernährungswissenschaften, Universität Wien Bakkalaureatsarbeit: „Ein Überblick über die Rolle des NMDA-Rezeptors in der Glutamat Exzitotoxizität".
09/1999–10/2004	Bundeshandelsakademie Hartberg, Matura

Berufserfahrung und Praktikum

12/2008–09/2012	SENNA Nahrungsmittel GmbH & Co KG Labormitarbeiterin (Geringfügig) Tätigkeitsbereich: Eingangskontrolle Rohstoffe (Sensorik), div. Analysen (Refraktion, NMR, Schmelz-/Rauchpunkt- und Farbbestimmung), Freigabe von Analysezertifikaten, Produktionskontrolle
04/2014–06/2015	Institut für Ernährungswissenschaften, Universität Wien; Praktikum

Persönliche Fähigkeiten und Kompetenzen

Sprachen:	Englisch in Wort und Schrift Italienisch Maturaniveau
Sonstiges:	WIFI Kurs Personalverrechnung 1 IBM SPSS Statistics 22

Wien, 01.10.2015