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1. Introduction

The present diploma thesis deals with the evaluation of the protective role of bilirubin in the organism. It was part of the FWF–Austrian Science Fund supported project “The physiological relevance of bile pigments. In vitro and in vivo evidence of antioxidant, anti-mutagenic and anti-carcinogenic potential and their mechanism of action”. The study was carried out at the Department of Nutritional Sciences in Vienna, Emerging Field Oxidative Stress and DNA Stability.

Yellow coloured bilirubin is an endogenous compound which is mainly derived from the degradation of red blood cells. The organism produces daily approximately 300 mg (0.7 mg/kg) of unconjugated bilirubin (UCB).

For a long time bilirubin was considered as a waste product of heme catabolism or even associated with illness or toxicity (eg. newborn jaundice).

Today there is mounting evidence indicating that bilirubin is more than a toxic waste product. It was shown that bilirubin has strong antioxidant and anti-mutagenic properties. Furthermore it was found that individuals with Gilbert’s syndrome (GS) have markedly improved protection against oxidative stress and lower incidence of diseases such as cardiovascular disease. GS is a mild, hereditary unconjugated hyperbilirubinemia ($> 17\mu\text{mol/L}$), occurring in approximately 3-17% of the population. Molecular genetic background of Gilbert’s syndrome is a polymorphism in the gene promoter for hepatic uridine diphosphate glucuronosyltransferase (UGT 1A1) which is responsible for regulation of bilirubin concentration in the body. The activity of UGT 1A1 is characteristically reduced in GS.

The organism is constantly exposed to free radicals which in excess can lead to oxidative stress and cause deleterious damage to important biomolecules such as DNA. Increased DNA damage or insufficient repair is a risk factor for several diseases such as cardiovascular diseases, diabetes and cancer. Therefore

research is focused to investigate the possible role of exogenous and endogenous antioxidants in prevention of disease.

Starting from this point, the present case-control study aimed at analysing the protective effect of mild elevated bilirubin towards oxidative stress and DNA damage in GS (n = 45) and a matched control group (n = 39). A further aim was to explore whether bilirubin possesses principle antigenotoxic functions.

DNA damage was analysed in peripheral mononucleated cells (PBMCs) ex vivo using the Comet assay; a widely used method to detect different DNA damage end points.

Due to the mild elevated bilirubin levels in GS the hypothesis was that the GS group would have improved resistance to DNA damage compared to the matched control group.

So far, there is no existing literature that proved the effect of elevated bilirubin concentrations in GS with the comet assay. Hence this thesis demonstrates results on this background for the first time.

Lab work, data collection and literature research were done in collaboration with my colleague Barbara Rittmannsberger. Thus the parts "*Literature survey*" and "*Materials and Methods*" are similar in both works.

2. Literature Survey

2.1. Oxidative stress

2.1.1. Free radicals

Free radicals are molecules or fragments of molecules, which are characterised by a high reactivity and instability. The reactivity of free radicals is caused by unpaired electrons in the outer shell, which is not a desirable state for the molecule [VALKO et al., 2006]. In order to regain stability, free radicals deprive the missing electron randomly from other molecules.

The most important class of free radicals in the biological system are radicals derived from oxygen, so called reactive oxygen species (ROS) and radicals derived from nitrogen, reactive nitrogen species (RNS) [DRÖGE, 2002]. Reactive oxygen species include radicals like the highly reactive hydroxyl radical ($\text{OH}^{\bullet}$), superoxide radical ($\text{O}_2^{\bullet-}$) and the non-radical hydrogen peroxide (H_2O_2), which is easily converted into a free radical [EVANS et al., 2004].

Reactive oxygen species in living organisms play dual roles, deleterious at high concentrations and sometimes beneficial roles only at low or moderate concentration. Beneficial physiological functions of reactive oxygen species are the defence against pathogens (eg. Phagocytosis), second messenger in a number of signalling pathways or the induction of mitogen response. Many responses mediated by ROS protect cells from oxidative stress. At high concentration ROS can cause damage to important cell structures, including lipids and membranes, proteins and DNA (deoxyribonucleic acid) by attacking them, comprising their natural function [VALKO et al., 2006].

The sources of ROS are either of endogenous or exogenous origin:

Endogenous factors are by-products of normal cell metabolism. ROS can be released from the mitochondria during oxidative phosphorylation. During oxidative phosphorylation, approximately 1-3% of electrons leak to oxygen forming the superoxide radical $\text{O}_2^{\bullet-}$ ($\text{O}_2 + \text{e}^- \rightarrow \text{O}_2^{\bullet-}$) [VALKO et al., 2006]. ROS

are moreover released from peroxisomes during degradation of fatty acids or from leukocytes during the defence mechanism against microbes by “respiratory burst” [VALKO et al., 2006; WU et al., 2004].

Exogenous sources for ROS production are life style factors like diet, alcohol, cigarette smoking, exercise and environmental factors like exposure to toxins and radiation [GIDRON et al., 2006].

2.1.2. Free radicals and Oxidative stress

As mentioned before, free radicals are not always deleterious at low or moderate concentrations. In healthy individuals, there is almost a balance maintained between oxidants and antioxidants [VALKO et al., 2006].

If the balance between oxidants and antioxidants is disturbed by excessive ROS production or deficient capacity of the antioxidant system, an undesirable state occurs that is termed “oxidative stress” [COLLINS, 2009]. See Figure 1

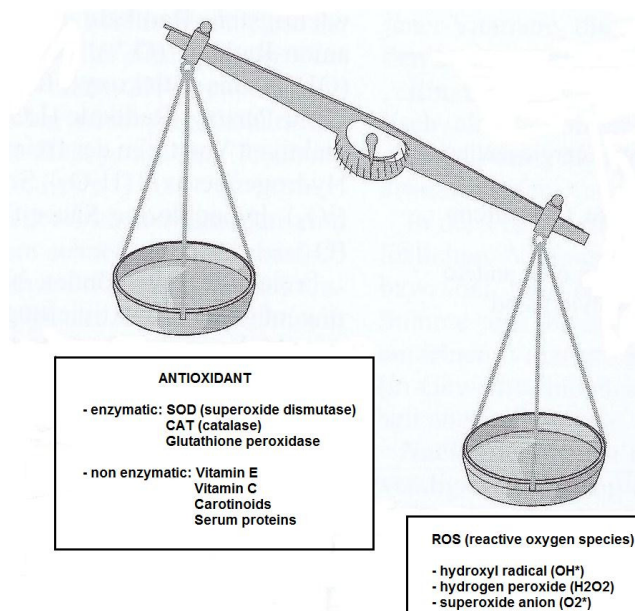


Figure 1: Oxidative Stress (modified after ELMADFA and LEITZMANN, 2004)

During oxidative stress in vivo ROS can attack important biomolecules. Cellular targets for oxidative modification include DNA, lipids and proteins. Which biomolecule is modified depends on several factors such as location of ROS production, relative ability for the biomolecule to be oxidised and availability of metal ions [EVANS et al., 2004].

Antioxidants can prevent or delay the oxidation of oxidisable substrates in vivo by detoxification of free radicals. This works by donation of hydrogen atom (electron) forming a stable compound and antioxidant-derived radical ($X^* + IH \rightarrow XH + I^*$) [NIKI, 2010]. Antioxidants are of physiological relevance and can either be enzymatic or non-enzymatic. Enzymatic antioxidant systems are, for example, the superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase (GSH-Px). Non enzymatic antioxidants can be, for example, vitamin E, vitamin C, carotinoids and serum proteins like uric acid and bilirubin. Some of these antioxidants are synthesised in the body, like glutathione, uric acid and bilirubin but some have to be provided alimentary, like vitamin C, E and β -carotin [ELMADFA and LEITZMANN 2004].

2.1.3. Oxidative stress and DNA damage

The DNA (deoxyribonucleic acid) is a relatively fragile biomolecule, which stores genetic instructions in living organisms. The DNA structure consists of two connected polymers which are made of simple units called nucleotides. Each nucleotide is composed of three parts: a phosphate, a sugar (deoxyribose) and a nitrogen base. Sugar phosphates link together by ester bonds forming the backbone of the double helix. The nitrogen bases pyrimidines (cytosine and thymine) and purines (adenine and guanine) bond with one another according to very strict rules (complementary base pairing) whereby the two single-strands are connected. Hence adenine pairs with thymine ($A \rightarrow T$) and guanine pairs with cytosine ($G \rightarrow C$) [SILVERSTEIN et al., 2008]. See Figure 2

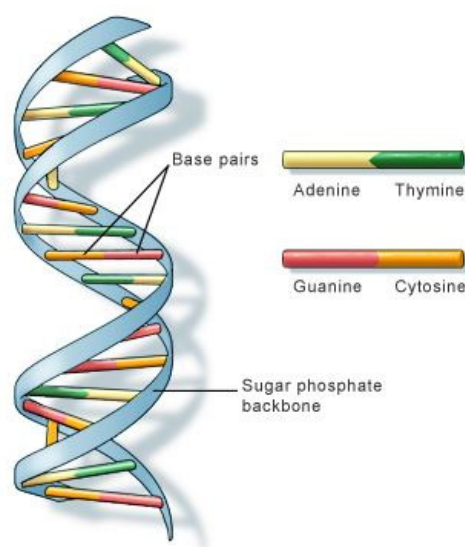


Figure 2: The basic structure of DNA [GHR, 2009]

The most important derivate of ROS, which causes damage to DNA is the hydroxyl radical ($\text{OH}^{\bullet}$). $\text{OH}^{\bullet}$ reacts closely to its site of formation and demonstrates the most reactive form of ROS which can react with all components of the DNA including purines, pyrimidines and DNA backbone [VALKO 2006]. In the body the $\text{OH}^{\bullet}$ - radical can be derived from H_2O_2 . The H_2O_2 molecule is less reactive and more stable compared to other ROS. However it plays an important role in oxidative damage since it easily diffuses through biological membranes reaching other cellular compartments and producing further cellular injury [MAYNARD et al., 2009]. At normal state H_2O_2 is converted into water and molecular oxygen by the action of antioxidant enzymes like glutathione peroxidase, superoxide dismutase (SOD) or catalase (CAT). If transition metals such as copper [Cu] or iron [Fe] are present, H_2O_2 can be reduced to the highly reactive $\text{OH}^{\bullet}$ - via the Fenton's reaction $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^{\bullet} + \text{OH}^-$ [REUTER et al., 2010]. See Figure 3

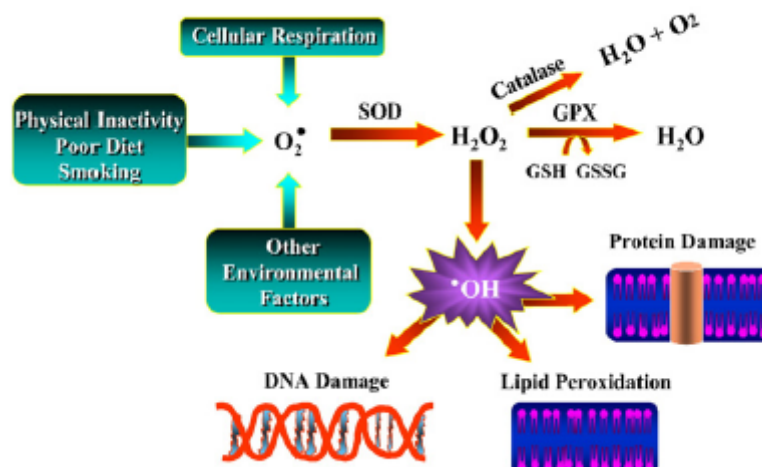


Figure 3: Generation and detoxification of ROS [ROBERTS and SINDHU, 2009]

The genome is under continuous attack from a variety of DNA–damaging agents leading to many different types of DNA lesions. To date, more than 100 oxidised DNA products have been identified [VALKO et al., 2006]. Damaged products of DNA include single-strand breaks (SSB), double-strand breaks (DSB), base-less sugar or AP (apurinic/aprimidinic) sites, DNA-Protein cross links and modified bases. DNA bases can be modified by oxidation or reduction, alkylation and deamination [EVANS et al., 2004; MAYNARD et al., 2009].

DNA damage can be devastating to the normal physiology leading to mutation and/or cell death. In order to avoid accumulation of DNA damage and its deleterious effect, damage needs to be repaired efficiently. The major repair pathways in mammalian cells are base excision repair (BER), nucleotide excision repair (NER), DSB repair and mismatch repair (MMR). BER is the predominant pathway for repair of ROS- generated lesions. NER removes bulky helix-distorting DNA lesions. In case of damage where both strands of the DNA are affected, DSB repair is used [MAYNARD et al., 2009].

2.2. Methods for measuring DNA damage

For detecting different endpoints of DNA damage a couple of methods exist. Comet assay is used to measure strand breaks and alkali labile sites (AP-sites). The inclusion of lesion-specific enzymes allows more specific and sensitive investigations of oxidative stress induced DNA damage. 8-OxodG is one of the major products of DNA oxidation and thus an important biomarker for oxidative stress induced DNA damage. 8-OxodG can be measured by various different methods like HPLC –ECD, GC/ MS, LC-MS/MS [COLLINS, 2009].

2.2.1. Oxidised Purines (8-OxodG)

Among the DNA bases purine and pyrimidine, guanine is most prone to oxidation. Throughout the oxidation of guanine, a hydroxyl radical is attached to the heterocyclic structure of guanine at the 8th position (C8-OH-adduct radical) and releases an H-Atom forming the oxidat ion product 8-OxodG (8-Oxo-7,8-dihydro-2'-deoxyguanosine) [WAGNER und JAHREIS, 2004]. The existing keto-enol tautomerism between the endproducts 8-OxodG and 8-OHdG (8-hydroxy-2'-deoxyguanosine) favours the 8-OxodG product (see Figure 4) [VALAVANIDIS et al., 2009].

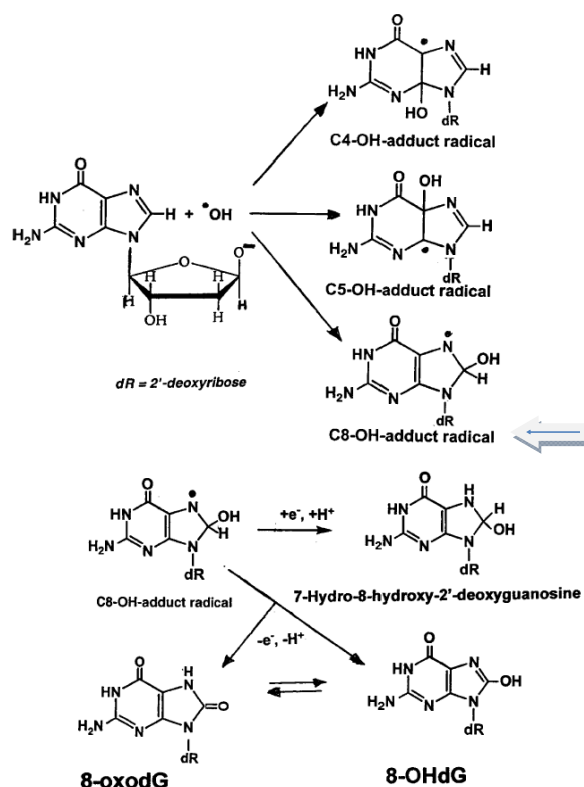


Figure 4: Forming 8-OxidG after reaction of 2'-deoxyguanosine with hydroxyl radicals [VALAVANIDIS et al., 2009]

8-OxidG is one of the most important lesions of DNA caused by free radicals. Therefore, 8-OxidG is often used as a biomarker for oxidative stress and it is also considered as a risk factor for oxidative stress related diseases such as cancer, atherosclerosis and diabetes [WU et al., 2004].

The lesion of 8-OxidG is particularly harmful, because it is able to structurally mimic thymine base and thus able to bypass replicative DNA polymerase which removes damaged bases before DNA replication. If 8-oxodG is not fully removed before replication, wrong base pairing occurs. Generally, adenine pairs with thymine and guanine pairs with cytosine, but due to oxidative modification of guanine, it pairs mistakenly with Adenine leading to guanine-thymine-transversion (G→T-transversion mutation) [DAVID et al., 2007].

8-OxodG was first detected by Kasai and Nishimura in 1984 with high performance liquid chromatography (HPLC) and electron capture detector (ECD). Consecutively different methods for measuring 8-OxodG were developed [WAGNER and JAHREIS, 2004].

➤ HPLC-ECD (high pressure liquid chromatography-electron capture detector)

A very sensitive (detection limit: 2 – 10 fmol) and correct method that has low costs of equipment and is commonly used. A disadvantage of this method is that it can only detect electrochemical active matters (like 8-OxodG) [WAGNER and JAHREIS, 2004] and suffers from adventitious oxidation during sample preparation [COLLINS, 2009; ESCODD, 2003].

➤ GC/MS (gas chromatography/mass spectrometry)

With gas chromatography and mass spectrometry most of the oxidised modifications are detectable (detection limit: 18 fmol). But to mention also some disadvantages it is a more expensive method and derivatisation is necessary. The obtained 8-OxodG levels are artificially increased what makes the interpretation of the results more complicated [WAGNER and JAHREIS, 2004].

Gas chromatography is therefore a not really suitable method to measure basal 8-OxodG in biological material [COLLINS, 2009].

➤ LC-MS/MS (liquid chromatography-mass spectrometry/mass spectrometry)

Liquid chromatography is theoretically the most powerful method with a detection limit of 7.5 – 10 fmol in contrast it is a very expensive method [WAGNER and JAHREIS, 2004]. But this method is also considered as relatively simple and sample pre-treatment is not absolutely necessary. LC-MS/MS method is often preferred because of its specificity and high sensitivity [LEE et al., 2010]. It is a method that can measure more than one product from DNA oxidation and is therefore often used for quantitative analysis of the oxidised nucleosides [HENRIKSEN et al., 2009].

2.2.2. Single cell gel electrophoresis (SCGE) (Comet Assay)

The Comet Assay, also called single cell gel electrophoresis (SCGE), was first carried out under neutral conditions by Östling and Johanson in 1984. The SCGE under neutral conditions was only for analysing double strand breaks. Therefore, a few years later in 1988 Singh et al. described a similar method but using alkaline conditions and a sensitive method for measuring both, single- and double-strand breaks in DNA, was found [DHAWAN et al., 2009].

The Comet Assay is named after the receiving image, which occurs after staining and looks like a “Comet”. The head of a Comet contains intact DNA whereas the tail is built by damaged or broken pieces of DNA [PIPERAKIS, 2009].

The **advantages** of the Comet Assay include

- the high sensitivity for detecting low levels of DNA damage,
- the small sample size (from 10000 to 50000 cells),
- the flexibility to use proliferating as well as non-proliferating cells,
- low costs,
- the easy and fast application,
- that results can be obtained on the same day,
- that fresh and frozen samples are suitable and
- virtually any eukaryotic cell population can be analysed. [DHAWAN et al., 2009; PIPERAKIS, 2009]

Some **limitations** and disadvantages of the Comet Assay might be

- that also non-oxidised DNA-damage is detectable,
- reproducibility is low, [WAGNER and JAHREIS, 2004]
- aneugenic effects (that are maybe a possible mechanism for carcinogenicity) and epigenetic mechanisms of DNA damage are not detected,
- single cell data (which may be rate limiting),
- small cell sample (leading to sample bias) and
- technical variability. [DHAWAN et al., 2009]

Although the Comet Assay may be less precise than for example HPLC the advantages of the Comet Assay predominate the disadvantages and the SCGE has been widely used in the last years in many different fields of research. By using this assay, it was possible to analyse the impact of oxidative stress in human diseases, to detect effects of environmental exposure to genotoxins and also to clarify the importance of antioxidants in our nutrition [COLLINS, 2009].

Procedure of the Comet assay:

The key principle of the method is the migration of the damaged DNA in an electric field at alkalised pH and measures the different intensities of the formed “comets” (see Figure 5).

For the procedure in the single cell gel electrophoresis cells (for example isolated peripheral human lymphocytes) are placed on a microscope slide with agarose. They have to be lysed with detergent of high salt to form nucleoids, which contain non-nucleosomal but still supercoiled DNA. Cells are exposed to an electric field in the electrophoresis and fragments of DNA can then migrate toward the anode and forming a “comet tail”. The amount of DNA in the tail represents the frequency of strand breaks and the comet assay is therefore a method for measuring DNA damage [COLLINS et al., 1997].

DNA strand breaks are then visualized with fluorescence microscopy after staining with a DNA-binding dye, such as ethidium bromide. Ethidium bromide is more efficient for double strand breaks than for single strand breaks [COLLINS, 2004].

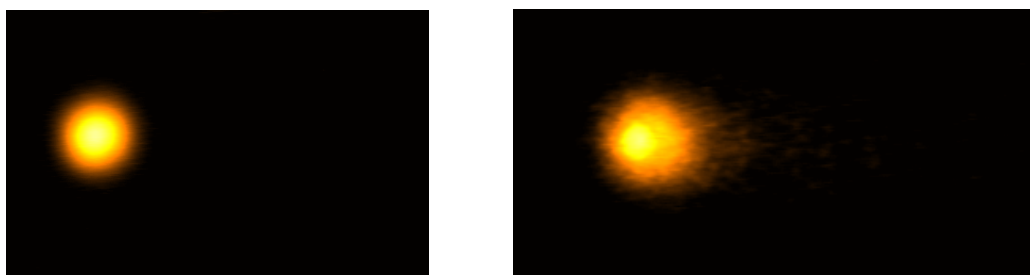


Figure 5: Photomicrographs of DNA from human lymphocytes after electrophoresis (left: no “comet”: no DNA migration; right: “comet”: damaged DNA, after treatment with ENDOIII)

A common additional step in the comet assay procedure is the treatment of cells with H_2O_2 . After incubation of cells with H_2O_2 mainly DNA strand breaks occur but also some oxidation in bases can occur. The receiving amount of DNA breaks is then characteristic for the changes in the resistance of the cells towards exogenous oxidative DNA damage [COLLINS, 2009].

2.2.3. Modified version of the Comet Assay

The comet assay under standard alkaline conditions ($\text{pH} \geq 13$) detects strand breaks (SSB and DSB) and alkali labile sites (AP-sites). AP-sites are locations on the DNA without DNA base. AP-sites occur as intermediates during DNA repair or arise spontaneously due to chemical change in bases or sugars. The detection of strand breaks and AP-sites therefore is not a specific index for oxidative stress in the organism [COLLINS, 2004; COLLINS et al., 2008].

In contrary to DNA strand breaks or AP-sites, oxidised bases are specific indicators of oxidative damage, reflecting the background damage caused by ROS in the cells. The value of oxidised bases can be detected with comet assay, by inclusion of lesion-specific enzymes after lysis. Hence, two particular DNA repair enzymes are frequently included; Endonuclease III (ENDO III) for detection of oxidised pyrimidines and formaminopyrimidine DNA glycosylase (FPG) which detects damaged purines including the major oxidation product of DNA damage 8 oxodG but also ring-opened purines or formamidopyrimidines (Fapy) [COLLINS 2004; COLLINS et al., 2008].

The enzymes recognise and remove the damaged base by glycosylase activity generating an AP-site. Subsequently associated AP-lyase cleaves the phosphodiester backbone at AP-site sites creating a nick in the DNA (See Figure 6). During the comet assay procedure strand breaks cause supercoiling of DNA to relax. Subsequently broken pieces of DNA migrate towards the anode during electrophoresis under alkaline conditions forming a typical comet tail whereas intact parts of DNA remain in the head [COLLINS, 2004; COLLINS et al., 2008].

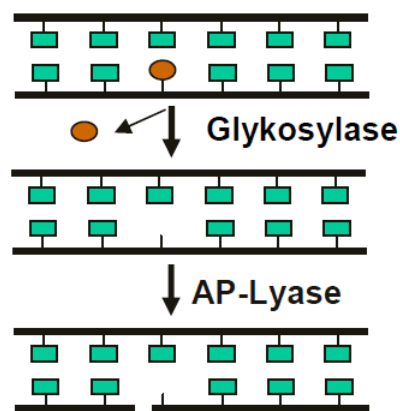


Figure 6: Function of FPG and ENDO III

In parallel with enzyme incubation, an extra slide is incubated with enzyme buffer alone serving as a control slide. The score from this slide is subsequently subtracted from the enzyme score to receive the net enzymatic sites. Most likely alkali labile sites (AP) are fully detected on the control slide. Therefore, net enzyme give precise and specific information about levels oxidised bases in the body [COLLINS, 2004; COLLINS et al., 2008].

The modified version of the comet assay is often used in order to investigate the relation between antioxidant levels in plasma and DNA damage or to attain changes DNA damage profile after supplementation with selected antioxidant-rich foods in human intervention trials [COLLINS, 2009]. Furthermore the modified version of comet assay is used to test the genotoxic potential of substances.

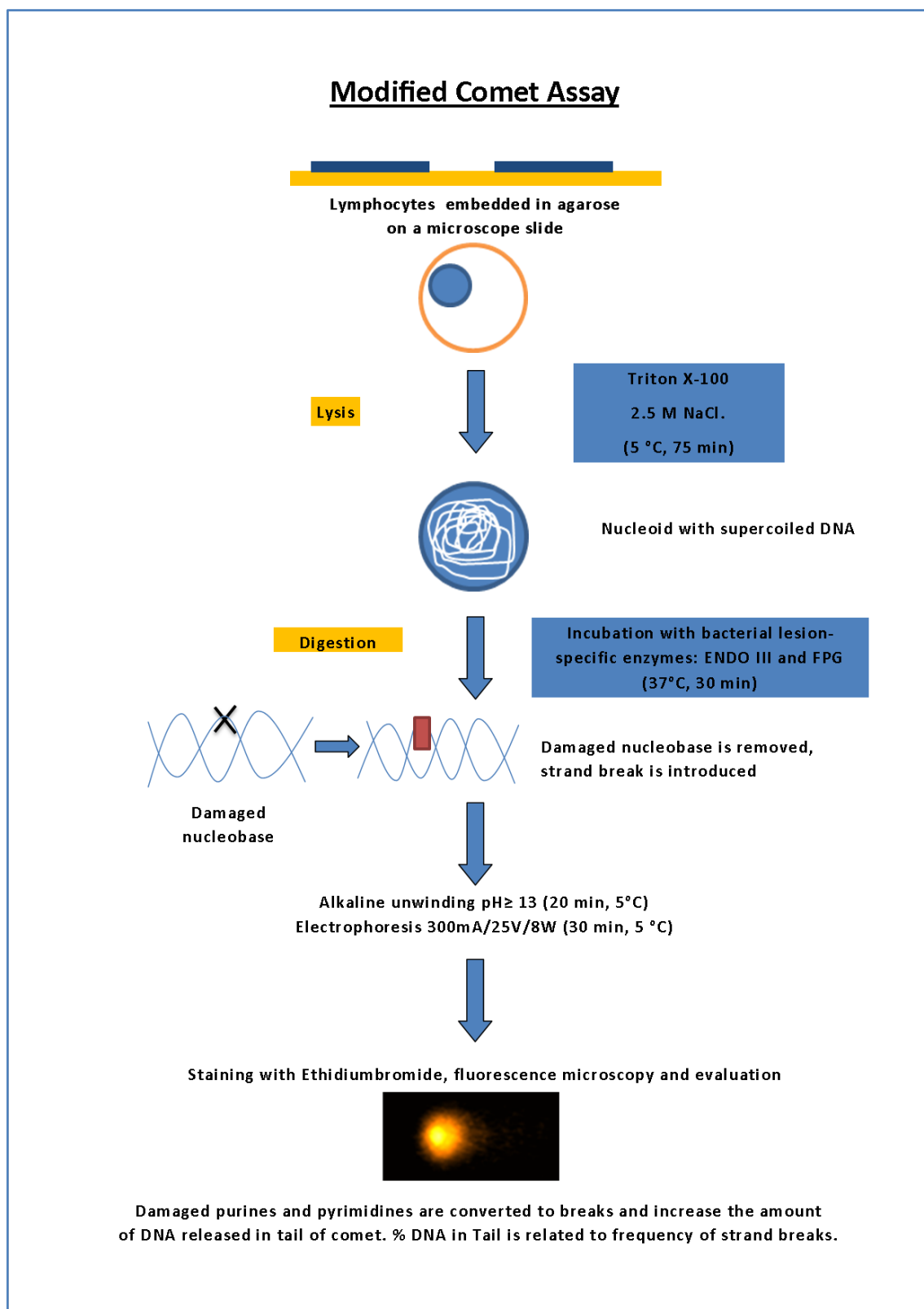


Figure 7: Methodology of modified version of comet assay. Detection of oxidised bases by inclusion of the bacterial lesion-specific enzymes FPG and ENDO III [modified after DUTHIE, 1999]

2.3. Bile pigments

2.3.1. Bilirubin structure

Bile pigments including yellow bilirubin and green biliverdin are natural endogenous compounds derived from catabolism of heme. Both molecules belong to the family of tetrapyrrolic compounds [VITEK and OSTROW 2009].

The fundamental structure of bilirubin composes four covalent connected pyrrole rings, forming an open, linear, tetrapyrrolic chain. The two outer pyrrole rings carry a polar lactam (-CO-NH) group and the central pyrrole rings carry a polar propione sidechain (-COOH) (see Figure 8) [VITEK and OSTROW 2009].

Bilirubin and biliverdin are flexible molecules, which can assume different molecular conformations. Bilirubin preferable has a folded ridge tile conformation, which is caused by the formation of intermolecular hydrogen bonding between carboxyl group and other polar groups in the molecule. This constellation is responsible for the lipophilic character of bilirubin at physiological pH, as the polar groups are in a way 'neutralised' [MC DONAGH 2010a]. In addition to its lipophilic character, bilirubin is susceptible to oxidation and very sensitive towards light; therefore investigations of bilirubin require minimal exposure to oxygen and light [VITEK and OSTROW 2009].

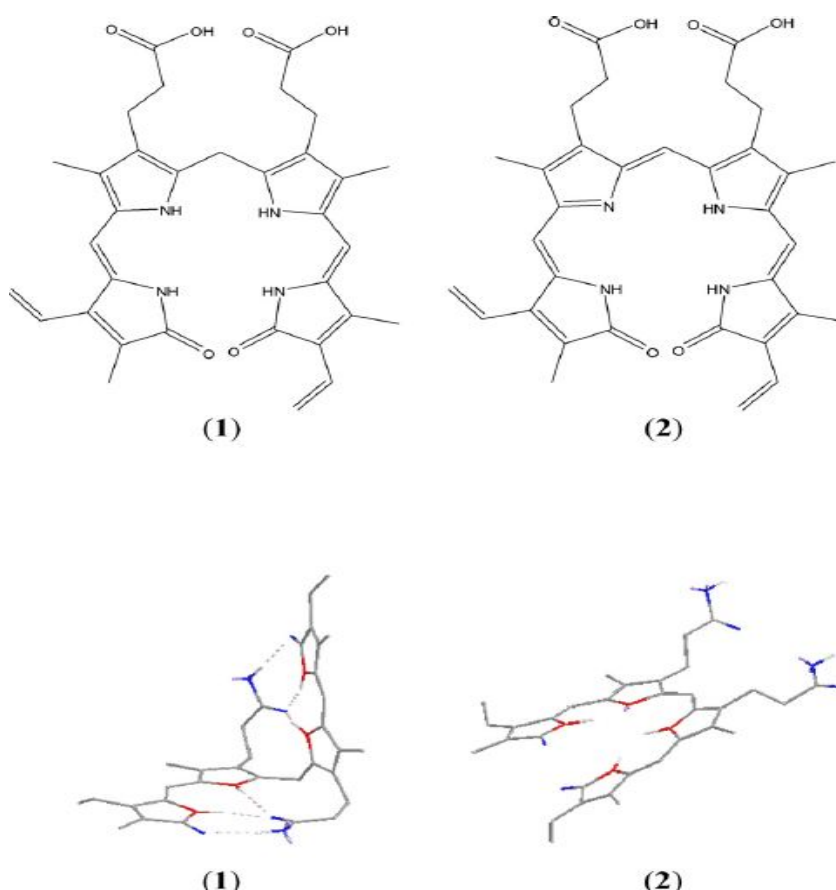


Figure 8: Two- and three-dimensional structures of (1) bilirubin and (2) biliverdin [BULMER et al., 2008b]

2.3.2. Heme Metabolism

Heme is well known as a component of hemoglobin in red blood cells. Furthermore a wide range of enzymes like cytochrome P450, catalase and peroxidase contain heme as a prosthetic group, which is released during their turnover [SEDLAK and SNYDER 2004].

Red blood cells have a limited life span and are decomposed in the reticuloendothelial system after 120 days whereby heme is released. Each day, approximately 300 mg (0.7 mg/kg) of unconjugated bilirubin (UCB) are produced which equates the amount of destructed heme in the body. 75 % of de novo UCB is derived by the destruction of senescent red blood cells and the remaining 25 % are derived from non-hemoglobin heme proteins [VITEK and

OSTROW 2009]. UCB is efficiently metabolised in the intestine by the action of anaerobic bacteria. The products of bacterial metabolism, so called urobilinoids, like stercobilin are mainly eliminated in the stool – excretion of yellow-orange coloured stercobilin 40-280 mg/d. Small proportions of urobilinoids undergo enterohepatic circulation, where a small part reaches the kidney and is excreted in the urine in the form of urobilin or urobilinogen approximately 0.64 mg/24h urine. In the case of high heme decomposition or liver damage the excretion of urobilin or urobilinogen in the urine is increased [PETRIDES, 2007].

The breakdown of heme is a multistep process and involves the two major enzymes, microsomal heme oxygenase (HO) and cystolic biliverdin reductase (BVR) (see Figure 9) [VITEK and OSTROW 2009]. Two genetic isoforms of heme oxygenase have been identified: HO-1 and HO-2. HO -1 is mainly expressed in reticuloendothelial cells and belongs to the family of heat shock proteins. HO-1 can be induced by stimuli which provoke oxidative stress (free oxygen radicals, free heme, heavy metals, bacterial lipopolysaccharides, hydrogen peroxide and ultraviolet light) whereas HO-2 is constitutive [VITEK and OSTROW 2009]. Bilirubin can be locally and temporally increased by specific induction of HO-1 in order to resist various situations of oxidative stress in short- and long-term [TOMARO and BATTLE 2002].

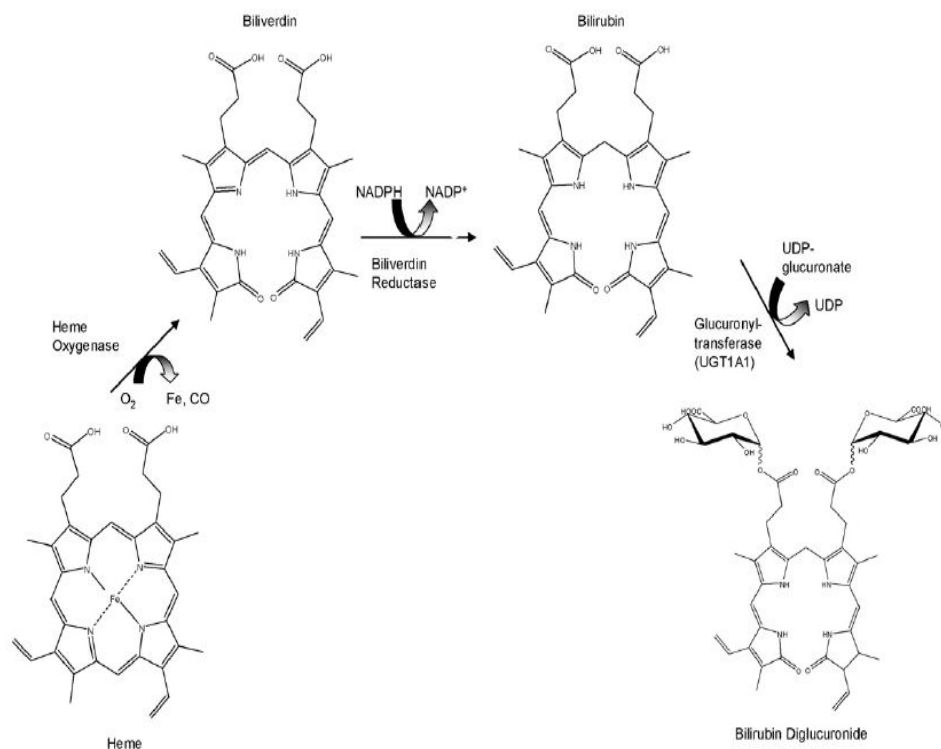


Figure 9: Metabolism of heme [BULMER et al., 2008b]

The first step of heme cleavage which is also the rate limiting step is mediated by HO Heme oxygenase which uses NADPH as a cofactor and oxidatively cleaves the heme ring at the α -carbon-bridge, forming the direct precursor of bilirubin, green coloured biliverdin (BV), carbonmonoxid (CO) and Iron (Fe^{2+}). Fe^{2+} can be reused and biliverdin is quickly reduced by biliverdin-reductase and NADPH to unconjugated bilirubin (UCB) [VITEK and OSTROW, 2009].

Biliverdin is only a transient intermediate. It is entirely and quickly reduced by biliverdin reductase to UCB. BVR occurs as a complex with membrane bound HO and in the cytosol facilitating the quick reduction of biliverdin [MC DONAGH, 2010a].

Colourfully the metabolism of bilirubin is displayed during bruising, where blue-red stained heme is followed by blue-green colour of biliverdin and by yellow colouration of bilirubin. Conjugated systems of double-bondings cause the colouration of these porphyrin molecules [KOOLMAN and RÖHM, 2002; BULMER et al., 2008b].

2.3.3. Bilirubin transport and excretion

Unconjugated bilirubin that is referred to as “indirect bilirubin” is poorly soluble in water. UCB needs to be tightly bound to serum albumin in order to be transported to the liver via the systemic blood circulation.

Before entering the liver, bilirubin is dissociated from its carrier protein, albumin, and taken up by the hepatocyte across the sinusoidal membrane. In the cytosol of hepatocytes bilirubin binds to ligandin with high affinity and is transported to the endoplasmatic reticulum (ER), where unconjugated bilirubin is converted into its water soluble form “direct bilirubin” by conjugation. Bilirubin is conjugated with glucuronic acid or sugar molecules (xylose or glucose) by UDP glucuronosyl transferase (UGT1A1) forming bilirubin monoglucuronide and diglucuronides [KAMISAKO, 2000; MC DONAGH, 2010a]. If this enzyme is not working properly, it can lead to an increase in bilirubin levels and bilirubin associated metabolic conditions like Gilbert’s syndrome, crigler-najjar syndrome or neonatal hyperbilirubinemia (See also chapter 2.4.) [PETRIDES, 2007].

The water soluble glucuronide derivates are excreted into the bile by the canicular ATP-dependent transport protein MRP2 (multidrug resistance associated protein 2) [MC DONAGH, 2010a].

In the intestine, bilirubin mono and di-glucuronide are hydrolysed by the action of anaerobic bacteria (β -Glucuronidasen). Bilirubin is reduced stepwise to stercobilinogen, which is a colourless compound that is partly oxidised to yellow-orange coloured stercobilin. Most of the end products of bilirubin metabolism are excreted with the stool, a small part is reabsorbed and undergoes enterohepatic circulation [PETRIDES, 2007; KOOLMAN and RÖHM, 2002].

2.3.4. Physiological concentrations of bilirubin

The physiological concentration of bilirubin in plasma is about 0.1 – 1.2 mg/dl (1.7 – 20.5 $\mu\text{mol/l}$) [PETRIDES, 2007]. Bilirubin levels vary significantly with gender, men in general show significantly higher serum bilirubin levels (0.72 mg/dl) compared to women (0.52 mg/dl) [ZUCKER et al., 2004]. “Unconjugated Bilirubin”, which is tightly bound to serum album, demonstrates the principle bile pigment in mammals and accounts 90 % of total circulating bilirubin in the body and less than 0.01 % of total bilirubin is unbound (free bilirubin, Bf) [VITEK and OSTROW, 2009].

2.4. Hyperbilirubinemia

As discussed before, bilirubin that is generated during the decomposition of erythrocytes is called unconjugated bilirubin. It is immediately bound to albumin in blood and transported to the liver. In the liver, unconjugated bilirubin is conjugated to glucuronic acid, consequently gets water soluble and is finally called conjugated bilirubin. A mutation in the gene promotor region of bilirubin-uridine-diphosphate-glucuronyl transferase (UGT1A1) can cause hyperbilirubinemia [THOMPSON, 2003].

Hyperbilirubinemia is a benign or malign condition that is caused by elevated bilirubin levels.

Mild rises in bilirubin levels can be caused by:

- Gilbert's syndrome: $>17.1 \mu\text{mol/l}$ unconjugated bilirubin [BULMER et al., 2008a]
- Rotor syndrome: increased (51.3 - 171 mg/dl) levels of conjugated bilirubin [MAIER, 2000]

Strong rises in bilirubin can be caused by:

- Neonatal hyperbilirubinemia (jaundice): conjugated and unconjugated bilirubin levels can be increased [THOMPSON, 2003]

- Crigler-Najjar-Syndrome: $>335 \mu\text{mol/l}$ unconjugated bilirubin [BULMER et al., 2008a]
- Dubin-Johnson Syndrome: $51 - 257 \mu\text{mol/l}$ conjugated bilirubin [GARTUNG and MATERN, 1999]

Bilirubin levels can also rise by haemolysis (increased breakdown of erythrocytes), hepatitis, choledocholithiasis, cirrhosis and chemotherapy [GARTUNG and MATERN, 1999].

2.4.1. Gilbert's syndrome

Gilbert's syndrome (GS) is also called "Morbus-Meulengracht" or "Icterus intermittens juvenilis". The clinical picture was first described by Gilbert and Lereboullet in 1901 and it is characterised by episodes of intermittent jaundice in the absence of haemolysis or liver disease [MUKHERJEE, 2011].

Gilbert's syndrome is the most common hereditary cause of increased bilirubin and is found in up to 3 – 17 % of the population [BULMER et al., 2008a]. Men are four times more often affected than women [GARTUNG and MATERN, 1999] and it is typically diagnosed for the first time during adolescence [KAPLAN et al., 2003].

Generally Gilbert's syndrome is considered as harmless in adults, although an incidental finding of hyperbilirubinemia may raise the possibility of liver disease [BOSMA et al, 1995].

Genetically the syndrome is characterised by a hereditary mutation in the gene promoter for hepatic uridine diphosphate glucuronosyltransferase (UGT1A1) [BULMER et al., 2008a]. The polymorphism in the UGT 1A1 gene promoter is commonly an additional TA base in the TATAA box. Affected individuals have 7 TA repeats, whereas non-affected individuals have 6 repeats. Due to the insertion of an additional TA base the activity of the UGT1A1 enzyme is decreased [KAPLAN et al., 2003]. As mentioned before, UGT1A1 is responsible

for glucuronidation of UCB and bilirubin clearance. In persons with GS a 30 % rest activity of the enzyme is found. Therefore, the circulating unconjugated bilirubin concentration is increased [BOSMA et al., 1995].

Bilirubin has an antioxidative potential in vitro [NEUZIL and STOCKER, 1994] and the ability to decrease oxidation of the plasma [FREI et al., 1988]. Results of a human study showed a better protection of serum oxidation in GS as for controls [BULMER et al., 2008a].

Elevated levels of serum bilirubin may play an important role in the prevention of ischemic heart disease. Due to the antioxidant capacity of bilirubin a possible protection of lipids and lipoproteins against oxidation is assumed. Therefore, elevated bilirubin levels, like in GS, might protect against atherosclerosis [YESILOVA et al., 2008].

Another study by Vitek et al. showed that high serum bilirubin levels are related to low urinary excretion of biopyrrins, an oxidative metabolite of bilirubin [VITEK et al., 2007]. Urinary excretion of biopyrrins is correlated with oxidative stress [SHIMOHARADA et al., 1998] and therefore, correlation with cardiovascular diseases (CVD) like heart failure [HOKAMAKI et al., 2004] or coronary spastic angina [MORITA et al., 2001]. These studies proved that people with GS are probably better protected against oxidative stress and CVD because of low urinary excretion of biopyrrins.

Schwertner et al. also described that high serum total bilirubin levels decrease the risk of coronary artery diseases [SCHWERTNER et al., 1994]. The first study on cardiovascular heart disease (CHD) risk and especially Gilbert syndrome patients was carried out by Vitek et al. in 2002. In this study, results showed that only 2 % of people with GS were found to have CHD compared to 12.1 % of the general population. First results concerning the decreased risk of CHD particularly in GS patients were shown [VITEK et al., 2002].

2.4.2. Neonatal hyperbilirubinemia

Neonatal hyperbilirubinemia is leading to jaundice, which means the yellowing of the skin and other tissues of a newborn infant. Cause of neonatal jaundice is an imbalance between UCB production and elimination [AHLFONS et al., 2009]. High serum bilirubin concentrations can be found around 2 – 5 days after birth and the bilirubin levels normally don't exceed a level of 90 $\mu\text{mol/l}$. In about 70 % of newborns this condition exists but bilirubin levels decline to a normal value after some days and therefore neonatal hyperbilirubinemia is mostly harmless [THOMPSON, 2003].

Normally jaundice in adults is harmless, but as the tissues protecting the brain are immature in newborns, neonatal jaundice can cause acute bilirubin encephalopathy (ABE) and this can lead to kernicterus or death. Consequences of kernicterus can be for instance athetoid cerebral palsy, auditory dysfunction, ocular movement disorders or dental enamel dyslasia. Phototherapy is a well-established method for enhancing UCB elimination in order to prevent ABE and kernicterus [AHLFONS et al., 2009].

2.4.3. Crigler-Najjar-Syndrome (CNS)

There are two syndromes of more severe unconjugated hyperbilirubinemia, namely the rare type 1, which often leads to neonatal death, and the more common and more harmless type 2. Both are caused by severe deficiency of the enzyme UGT1A1 [THOMPSON, 2003].

In type 1, the disease is autosomal recessive inherited with an incidence of 1:10000 [GARTUNG and MATERN, 1999] and in neonates this can rapidly cause jaundice in the first days with an unconjugated bilirubin level from 350 to 950 $\mu\text{mol/l}$. A correct treatment is necessary to avoid development of kernicterus or brain damage.

Crigler-Najjar-Syndrome type 1 desires a whole body blue-light phototherapy for 16 h daily. Another rehabilitation would be plasmapheresis until a liver transplantation is possible, as a definitive cure. [THOMPSON, 2003] Another standard treatment for Crigler Najjar Syndrome would be the Phenobarbital therapy. As in many of these patients a defect of the production of the enzyme UGT1A1 occur, an addition of Phenobarbital can stimulate enzyme production and therefore, helps to remove bilirubin. However, not all patients respond positively to Phenobarbital therapy as a reduced enzyme production is not the only cause of illness [<http://carmonet.50webs.com/CNS/phenobarb.html>; access date: 16.06.2011].

Crigler-Najjar-Syndrome type 2 can either be inherited dominant with incomplete penetrance or autosomal recessive. On the contrast to type 1 bilirubin levels are lower ($<350 \mu\text{mol/l}$) [THOMPSON, 2003] and the rest activity of the UGT1A1 can be $<10 \%$ compared to normal [GARTUNG and MATERN, 1999]. Brain damage doesn't occur and persistent mild jaundice is only noticed in childhood [THOMPSON, 2003].

Drugs like penicillin or salicylates have to be avoided in people with Crigler-Najjar-Syndrome, because they have the ability to displace unconjugated bilirubin from albumin [THOMPSON, 2003].

2.4.4. Dubin-Johnson Syndrome and Rotor Syndrome

Dubin-Johnson and Rotor Syndrome are rare forms of non-haemolytic and autosomal recessive disorder with conjugated hyperbilirubinemia without cholestasis [THOMPSON, 2003].

In the Dubin-Johnson Syndrome bilirubin levels are between $51 - 257 \mu\text{mol/l}$ and usually it is asymptomatic but may be diagnosed by incidental finding in early infancy. Generally this syndrome is harmless but jaundice can occur sometimes during pregnancy or intake of hormonal contraceptives [GARTUNG and MATERN, 1999].

A typical indication of Dubin-Johnsons Syndrome is an accumulation of black pigments in the liver hence, at laparoscopy the liver appears strikingly black. Another diagnostic finding is that the urinary coproporphyrin excretion is either increased or decreased [THOMPSON, 2003].

The Rotor-Syndrome is a disorder similar to the Dubin-Johnson Syndrome with unknown origin and unpigmented liver cells. The main symptom is a non-itching jaundice and elevated conjugated bilirubin serum levels [THOMPSON, 2003].

2.5. Protective role of bile pigments in literature

2.5.1. First findings and bilirubin as antioxidant

Throughout a long period, the best property attributed to bilirubin was its role as a waste product of heme catabolism that needed to be excreted from the body. Bilirubin at severe high concentrations can be neurotoxic causing acute or chronic neurological damage [BULMER et al., 2008b].

The first evidence that bilirubin is more than a toxic by-product, was delivered by Bernhard in the 1950s. In his experiment only small quantities of bilirubin stabilised Vitamin A or β -carotene and effectively protected lipids, especially linoleic acid from oxidation [BERNHARD et al., 1954].

Later on it was shown that bilirubin can inhibit lipidperoxidation due to a synergistic action with membrane bound Vitamin E [STOCKER and PETERHANS, 1989]. Unconjugated bilirubin was also shown to synergise with α -tocopherol to prevent lipoprotein lipid peroxidation. Furthermore, bilirubin inhibited the formation of cholesterylester hydroperoxide (CE-OOH), the oxidation product of LDL, by scavenging alpha tocopherol radical (TO) in LDL [NEUZIL and STOCKER, 1994].

In the last two decades the beneficial role of bile pigments gained high interest and much research was done in order to confirm their physiological importance.

It was demonstrated, that bilirubin has anti-mutagenic, anti-viral, anti-inflammatory and anti-proliferative properties [BULMER, 2008; ZUCKER et al., 2004].

More recent investigations showed that bilirubin and biliverdin have the ability to suppress lipopolysaccharide (LPS) - induced oxidative burst in isolated human neutrophils [JANSEN et al., 2010].

Sedlak and co-workers investigated the complementary effect of bilirubin and reduced glutathione (GSH) two prominent endogenous antioxidants. They indicated that lipophilic bilirubin protects mainly against lipid peroxidation of cell membranes, whereas GSH which is a hydrophilic compound, prevents the oxidation of water soluble proteins [SEDLAK et al., 2009].

It was suggested that nanomolar concentrations (10 nM) of BR can protect cultured cells from an excess of H_2O_2 [DORÉ et al., 1999].

The exact working mechanism of bilirubin as an antioxidant remains unclear and more research is needed in this area. One suggestion independent from the structural aspects of bilirubin is that the antioxidant action of bilirubin originates from the redox-recycling with biliverdin. Thus, when bilirubin acts as an antioxidant, bilirubin is oxidised to biliverdin, which is subsequently reduced back to bilirubin by BVR. This mechanism amplifies the antioxidant property of bilirubin as it is regenerated over and over again. Thus, enabling low concentrations of bilirubin to cope with high concentrations of reactive oxygen species [Mc DONAGH, 2010b]. This was chemically shown by incubation of bilirubin-albumin with 2,2' -azobis (2-amidinopropane) hydrochloride (AAPH), where BV was formed during peroxy radical attack [BARANO et al., 2002].

The recycling between bilirubin and biliverdin seems to be a plausible explanation for the strong antioxidative potential of bilirubin. Nevertheless some researchers are very critical with this hypothesis. Maghzal and co-workers for instance could not confirm the hypothesis that oxidation of bilirubin specifically delivers biliverdin [MAGHZAL et al., 2009]. Moreover the in vitro experiments of

Jansen and co-workers speak against this theory. In their study, the conversion of BR to BV was less than 5% for all oxidants tested [JANSEN et al., 2010].

Stocker also implicates structural aspects of bilirubin. Bilirubin contains a system of conjugated double bonds and a pair of reactive hydrogen bonds. The latter can neutralize free radicals via H- donation [STOCKER, 2004].

2.5.2. Albumin-bound bilirubin

A strong evidence exists that albumin bound bilirubin protects proteins against oxidative damage by several types of reactive oxygen and nitrogen species (RONS). Already in 1987, an in vitro study by Stocker et al. demonstrated that free fatty acids, especially linoleic acid, transported by albumin can be protected from oxidative damage. The albumin-bound bilirubin increases with specificity and reactivity against RONS and this result in an elevated protection of plasma proteins and lipids. In this in vitro study the antioxidative effect of bilirubin in plasma was also compared with uric acid and vitamin C. The results indicate that bilirubin has the same antioxidative effect as uric acid but has a weaker effect compared to vitamin C. But this was an in vitro study and still more investigations would be needed to demonstrate this theory [STOCKER et al., 1987].

To sum up, there is of course already some evidence that proves the antioxidant capacity of bile pigments in vitro. In fact, however, it is necessary to observe whether this action remains also in vivo situations.

There are only a few in vivo studies illustrating the protective role of bile pigments. One of these was published by Dennery and co-workers (1995). In this study hyperbilirubinemic Gunn rats were exposed to a hyperoxia (>95 % O₂) atmosphere for 3 days. Oxidative markers were inversely correlated to serum bilirubin concentrations [DENNERY et al., 1995].

Another important work demonstrating the antioxidative role of bilirubin in vivo was published by Noriega et al [2003]. They examined the effects of bilirubin (5 mmol/kg body weight) in aminolevulinic acid (ALA) induced liver damage in Wistar rats by intraperitoneal injection. This study showed that bilirubin can act as an efficient antioxidant against oxidative cell damage induced by ALA [NORIEGA et al., 2003].

2.5.3. Bilirubin as protector against cardiovascular diseases

Bulmer et al. (2007) investigated bilirubin as protector against cardiovascular diseases in patients with GS. The study demonstrated that people with GS shown an improved resistance to serum oxidation and thus show a reduced prevalence of cardiovascular disease compared to a control group. In conclusion, it was suggested that higher levels of bilirubin can increase the status of circulating antioxidants and improve resistance to serum oxidation and may protect from prevalence of developing cardiovascular diseases (CVD) [BULMER et al., 2008a].

Some other studies showed that there is a low prevalence of coronary artery disease or ischemic heart disease in people with GS or generally in people with higher bilirubin levels. There is probably an increasing serum antioxidant capacity in people with higher serum bilirubin levels that prevent the occurrence of ischemic heart diseases [VITEK et al., 2002; SCHWERTNER et al., 1994]. A negative correlation between serum bilirubin levels and atherosclerosis was shown and these results might indicate the importance of bilirubin in the prevention of cardiovascular diseases or in general oxidative-stress mediated diseases [NOVOTNY and VITEK, 2003].

2.5.4. Bilirubin and DNA damage and cancer

Overwhelming amounts of reactive oxygen species of endogenous or exogenous origin can cause damage to DNA. DNA lesion that remains unrepaired, increase the prevalence of mutations which play a key role during carcinogenesis.

Bilirubin was shown to be a strong antioxidant that can prevent the oxidation of several biomolecules. There are only few studies that show the protective effect of bilirubin towards DNA oxidation and its role in the possible prevention in the etiology of cancer. The few numbers of studies, which investigate the effect of bilirubin levels, deliver conflicting results [ZUCKER et al., 2004].

In the study of Asad et al. (2000) it was shown, that 50 μ M of bilirubin were able to reduce the amount of oxidative DNA single strand breaks in calf thymus DNA induced by L-Dopa-Cu(II) by 50 %. A concentration of 20 μ M bilirubin reduced the generation of hydroxyl radicals by 30 %. The results of this study suggest that any increase from normal bilirubin levels under conditions of oxidative stress would be able to afford protection of cellular DNA from L-Dopa-Cu(II)-mediated damage [ASAD et al., 2000].

Two years later (2002) the same research group of Asad and co-workers showed, that bilirubin has pro-oxidant effects upon DNA due to its ability to bind with the transition metal ions Cu(II) and in the following form a bilirubin CU(II)-DNA complex which causes oxidative DNA cleavage [ASAD et al., 2002].

Throughout a study by Rao et al. additionally the effect of bilirubin on DNA damage was investigated using the comet assay. Cells treated with bilirubin showed a typical comet tail indicating that bilirubin causes DNA damage. This study showed that bilirubin has anti-cancer activity as a prooxidant [RAO et al., 2006].

In a Belgian prospective study the correlation of serum bilirubin and 10-year mortality risk including all cause, cardiovascular and cancer mortality was assessed in a Belgian population. The results showed that men with higher

serum bilirubin levels had a significant reduced risk of non-lung cancer mortality. In women the same trend was observed but lacking statistical significance. There was no significant association detectable between serum bilirubin and cardiovascular mortality in neither men nor women [TEMME et al., 2001].

In the USA, a prospective study on the potential chemopreventive function of bilirubin was investigated by Zucker et al. (2004). Data of the study showed that bilirubin levels vary significantly with gender, race and smoking status. Men have higher bilirubin levels compared to women. Serum bilirubin levels were lower in active smokers compared to non-smokers. Total serum bilirubin levels were also lower in subjects which reported prior nondermatological cancer in comparison to subjects who did not have a history of nondermatological malignancy. The results of the study showed that an increase in serum bilirubin by 1.0 mg/dl leads to a decreased prevalence of colorectal cancer (odds ratio: 0.257). The inverse relationship between serum bilirubin and colorectal cancer was more robust for women. There was also an inverse relationship between serum bilirubin and non-gastrointestinal cancers but at a lower level (odds ratio: 0.809) [ZUCKER et al., 2004].

Very recently a cohort study examined the relationship between serum bilirubin levels and respiratory diseases and lung cancer among adults in UK. Incidence of lung cancer was assessed and the results showed that an increase in bilirubin level of 0.1 mg/dl was accompanied by an 8 % decreased lung cancer incidence in men and 11 % decreased lung cancer incidence in women [HORSFALL et al., 2011].

Bilirubin is considered as a strong endogenous antioxidant and it is discussed that slightly elevated bilirubin levels have beneficial functions for health. With the present study we aimed at investigating the protective effect of bilirubin at DNA level and to measure its protective effect using the comet assay. By doing so, the DNA damage of individuals with GS (and hence elevated levels of bilirubin) and a control group (with normal levels of bilirubin) was compared.

3. Material and Methods

3.1. Study design and in- and exclusion criteria

In the present case-control-study 45 subjects with Gilbert's syndrome (GS) and 39 healthy persons as control group (C) were included. Due to their UCB level very close to 1 mg/dl 16 persons could clearly not be allocated to one of the groups. There was a difference between first and second blood sampling in reaching threshold values. For a clear allocation an additional blood sampling or determination of genotype would be needed. The aim of the study was to observe whether people with GS have a lower DNA damage than controls. Cases and controls were matched for age and sex. The experimental design is summarised in Figure 10.

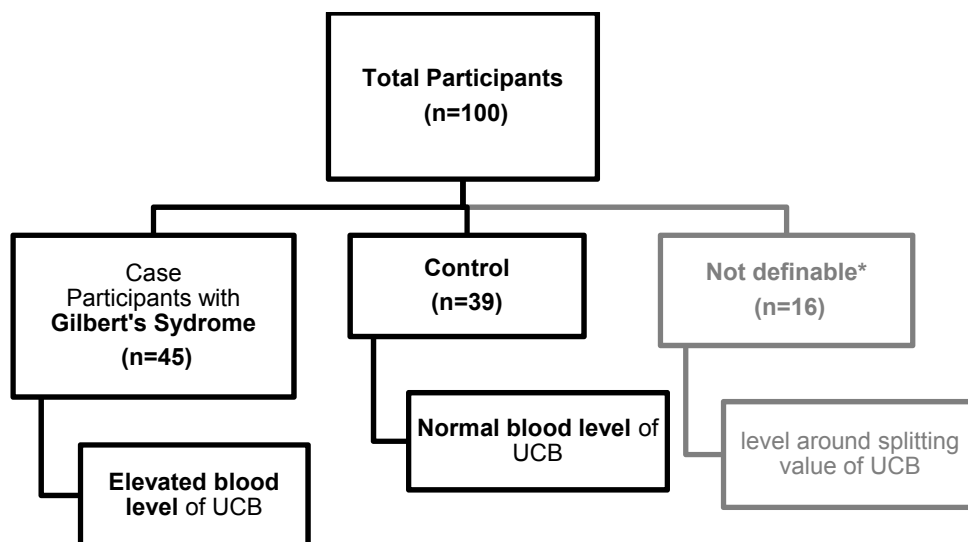


Figure 10: Illustration of the experimental design

Participants were recruited by information-sheets in different buildings and places (hospitals, universities, pharmacies ...). In order to assess relevant parameters, blood was withdrawn from all participants at the beginning of the study at the General hospital (AKH). Inclusion and exclusion criteria were checked in combination with questionnaires.

A second blood sampling was performed at the AKH for the main parameter and besides urine collection, buccals cells were obtained.

In order to carry out the Comet assay, one of the main methods for measuring DNA damage in peripheral blood mononucleated cells (PBMCs), the blood samples were immediately transported to the Department of Nutritional Sciences at the “University of Vienna”. By applying the Comet assay it was observed whether people with GS are better protected against oxidative DNA damage than controls with lower bilirubin levels.

Inclusion criteria:

- Age: 20-80 years
- total bilirubin level > 1.2 mg/dl [20.52 μ mol/l] (GS)
- unconjugated bilirubin level > 1.0 mg/dl [17.1 μ mol/l] (GS)
- total bilirubin level <1.2 mg/dl [20.52 μ mol/l] (Control)
- unconjugated bilirubin level <1.0 mg/dl [17.1 μ mol/l] (Control)
- Gamma-Glutamytransferase (GGT) <39 U/l (women) <66 U/l (men)
- Alanin Aminotransferase (ALT) <35 U/l (women) <50 (men)
- Aspartat Aminotransferase (AST) <35 U/l (women) <50 U/l (men)
- Moderate fitness

Exclusion criteria:

- < 20 years or > 80 years
- Cardiovascular diseases
- Hepatitis B/C
- Liver diseases
- Cholelithiasis
- Haemolysis
- Heart diseases
- Chronical kidney disease

- Acute tumor
- Diabetes Mellitus
- Supplementation of antioxidant within the last 4 weeks before the first blood sampling
- No intake of drugs which affect liver parameters (eg. Probenecid, Rifampicin) within the last 5 weeks before the first blood withdrawal
- Smokers >5 cigarettes a day
- People with organ transplantation
- Competitive athletes (>10 hours training per week)

3.1.1. Isolation of peripheral blood mononucleated cells

- Whole blood was sampled by venipuncture in heparinized tubes.
- 7 ml of anticoagulant whole blood was filled in 12 ml Leucosep tubes.
- Tubes were centrifuged at 1000 rcf for 15 Min. at 16 °C (without brake).
- The enriched fraction of lymphocytes, in particular peripheral blood mononucleated cells (PBMCs) were gently pipetted into a 15 ml centrifuge tube and filled up with 4 °C cold PBS-Buffer to a total volume of 15 ml. From here all steps were executed on ice.
- Tubes were centrifuged at 304 rcf for 15 Min. at 22 °C (with brake).
- Supernatant was discarded and PBMC pellet was washed in 10 ml of PBS-buffer.
- Tubes were centrifuged at 304 rcf for 10 Min. at 22 °C (with brake).
- Supernatant was gently discarded and PBMC pellet was suspended in 1 ml of PBS for cell counting.

3.1.2. Cell counting determination

To ensure a sufficient cell number per slide and to prevent overlapping of cells (if cell concentration is too high), a defined cell concentration of 1×10^6 cells/ml is necessary for the comet assay procedure. Furthermore a total suspension volume of 250 μ l is needed. For cell count determination the automated cell counter “Countess” from Invitrogen was used.

A cell suspension of appropriate concentration (approximately 10^6 cells/ml) was prepared; therefore 20 μ l of cell suspension was diluted in 60 μ l PBS-buffer in a cup. 10 μ l cell suspension was mixed with 10 μ l 0.4 %Trypan blue (1:1). Finally, 10 μ l were transferred in a chamber on a slide and inserted into the “Countess Invitrogen”.

3.2. General principles of the Comet assay

Comet assay is a widely used method for measuring DNA damage. The cells are embedded in agarose on a microscope slide. Lysis is used for removing membranes, cytoplasm and nucleoplasm. After treatment with H_2O_2 DNA strand breaks are occurring and different enzymes are used to detect oxidised pyrimidines (ENDOIII) and oxidised purines (FPG).

In an electric field at an alkalised level (electrophoresis), damaged DNA migrates faster than the compact and undamaged DNA. After staining, the different intensities of the formed “comets” can be evaluated with a fluorescence microscope [COLLINS, 2004].

3.3. Comet Assay

3.3.1. Equipment for Comet Assay

Table 1: Equipment for Comet Assay

Material	Company	Product number
Analytical balance	Mettler	AT201
Balance	Mechatronics Austria	41107690
Calibration solution	HANNA instruments	
Centrifuge	Eppendorf	5417R
Computer		
Countess-automated cell counter	Invitrogen	
Countess-cell counting chamber slides	Invitrogen	
Drying chamber	Memmert GmbH	Modell 500; D06061
Electrophoresis	Biozym	Serial no: C5 08091040; Stock Code: CSL-10M40
Examination gloves	Microflex	
Heating Plate/Magnetic stirrer	Heidolph	MR 3001K
Microscope	Zeiss, Hitachi	
Microscope slides	VWR	ECN 631-1551
Microscope Cover glasses (22x22 mm, 20x20 mm)	VWR	ECN 631-1570
Microwave	Elta	MW 170 (17MXS1); 320V 50Hz 700W
Moist box		
Nitrile Examination gloves	Mikroflex	9953.01
pH-meter	Metrohm	691 pH-meter
Staining dishes	Hellendahl	2460
Silver tray		
Water bath (37 °C, 55 °C)		

3.3.2. Reagents for Comet Assay

Table 2: Chemicals and Reagents for Comet Assay

Reagents	Company	Product number
Albumin from bovine serum (BSA)	Sigma Life science	A2153-50G
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma Life science	D5837
Ethylenediamine tetraacetic acid (EDTA)	Sigma Life science	E6758
Ethidium bromide aqueous solution	Sigma Life science	E1510
Hydrochloric Acid (HCL)	Riedel-de Haen	
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	Sigma Life science	H3375
Hydrogen peroxide solution 30 wt % solution in water (H ₂ O ₂)	Sigma Life science	
Potassium chloride (KCl)	Riedel-de Haen	31248
Potassium hydroxide (KOH)	Sigma-Aldrich	60370
Sodium Chloride (NaCl)	Sigma-Aldrich	71380
Sodium Hydroxide (Pellets) (NaOH)	Riedel-de Haen	06203
Trizma base (Tris)	Fluka Analytical	93350
Triton X-100	Sigma-Aldrich	
Trypan blue stain 0.4%	Invitrogen	T10282
Ultra Pure Agarose (NMA)	Invitrogen	153001
Ultra Pure LMP Agarose (LMA)	Invitrogen	144556

3.3.3. Manufacturing process and storage of solutions for Comet Assay

The reagents, used for the comet assay process, were manufactured according to the protocol “Protocol for the single cell gel electrophoresis/Comet Assay for rapid genotoxicity assessment” published by Dhawan et al (w.y.).

Alkaline electrophoresis buffer (pH > 13)

24 g NaOH (0.3M)

580 mg EDTA (0.001M)

NaOH and EDTA were dissolved in 2 l double distilled H₂O. PH should be higher than 13 and can be adjusted with NaOH. Stored at 4 °C in the cooling chamber.

Enzyme reaction buffer for FPG and ENDOIII

9.53 g HEPES (40 mM)

7.46 g KCl (0.1 M)

0.15 g EDTA (0.5 mM)

0.2 g BSA (0.2 mg/ml)

HEPES, KCL, EDTA and BSA were dissolved in 1 l double distilled H₂O and adjusted to pH 8.0 with KOH (1M). This buffer could also be made as 10x stock (adjusted with KOH (5M), be filled in aliquots and stored at -20 °C. Before usage the pH has to be controlled and adjust to 8 with KOH (5M).

H₂O₂ Stock solution

103 µl Hydrogen peroxide solution (30%)

Double distilled H₂O

103 µl of the hydrogen peroxide solution was mixed with 10 ml double distilled H₂O to receive a 0.1M stock. Before usage 80 µl of this 0.1M stock were mixed with 79.92 ml double distilled water and stored at 4 °C in the cooling chamber.

Lysis Solution (pH = 10)

146.1 g NaCl (2.5M)

29.2 g EDTA (0.1M)

1.211 g Tris (10mM)

Double distilled H₂O

Triton X-100

NaCl, EDTA and Tris were dissolved in 1 l double distilled H₂O and the pH was adjusted to 10 with NaOH (10M). Solution was stored at room temperature. Triton X-100 (1 ml per 100 ml) was added immediately before use and then the solution was stored at 4 °C in the cooling chamber.

NaOH (10N)

400 g NaOH

Double distilled H₂O

400 g NaOH were dissolved in 1 l double distilled H₂O to receive a 10N NaOH solution. Stored at room temperature.

Neutralization buffer (pH = 7.5)

48.44 g Tris base (0.4M)

Double distilled H₂O

48.44 g Tris base (0.4M) were dissolved in 1 l double distilled H₂O and the pH was adjusted to 7.5 with 37 % HCl. Buffer was stored at 4 °C in the cooling chamber.

Normal Melting Agarose (NMA)

1000 mg NMA

Double distilled H₂O

1000 mg NMA were dissolved in 100 ml double distilled H₂O, heated in microwave and stored at 4 °C in the cooling chamber. Before usage the agarose was heated up again in the microwave until it was liquefied.

Low Melting Agarose (LMA)

800 mg LMA

80 ml PBS

800 mg LMA were dissolved in 80 ml PBS, heated in microwave and stored at 4 °C in the cooling chamber. Before usage the agarose was heated up again in the microwave until it was liquefied.

Ethidium bromide solution

20 µl ethidium bromide solution

Double distilled H₂O

20 µl of ethidium bromide solution was mixed with 10 ml double distilled H₂O to receive a concentration of 20 µg/ml. Stored at 4 °C in the fridge.

3.3.4. Protocol for Comet Assay

The Comet Assay was carried out according to the advices of “Protocol for the single cell gel electrophoresis/Comet Assay for rapid genotoxicity assessment” published by Dhawan et al. (w.y.).

The general activity during the comet assay is illustrated in Figure 11.

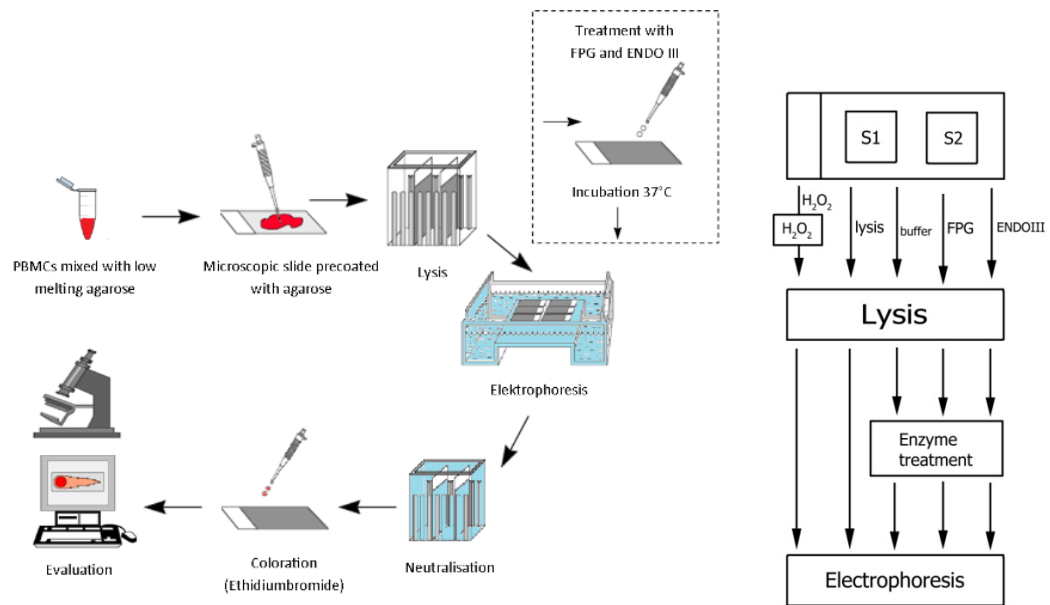


Figure 11: General activity of comet assay (left) and specific gels treatment (right)

For lysis 'no treatment', FPG, ENDOIII and buffer 3 slides were prepared and 2 slides for H_2O_2 . Each slide contained two gels and 2 subjects (S1, S2) all together 14 slides were prepared for 2 participants.

Slides treated with lysis stayed in lysis solution during the entire time, whereas H_2O_2 slides were first treated with hydroperoxide and subsequently kept in lysis solution until electrophoresis. Slides treated with buffer, FPG or ENDOIII stayed in lysis solution (at least 75 minutes) until incubation with enzymes started. See also Figure 11.

Slide preparation:

- Prepared NMA was heated 3 times in the microwave until it was completely liquefied and dissolved. Jar with dissolved NMA was transferred into a water bath and kept at 55 °C.
- Slides were then dipped in the NMA-agarose and excess agarose was drained off. The backs of the slides were then wiped with a paper towel. Slides were air dried overnight, before they were stored at room temperature.

Embedding cells in agarose: (see Figure 12)

- Prepared LMA was heated 3 times in the microwave until it was completely liquefied and everything was properly dissolved. Subsequently LMA was put into a warm water bath and kept at 37 °C.
- Slides precoated with NMA were labelled with subject number and the kind of treatment: "lysis", "H₂O₂", "buffer", "FPG" and "ENDOIII".
- 30 µl of cell suspension (with a concentration of $c = 1 \times 10^6$ cells per ml) was resuspended in an extra cup with 140 µl LMA.
- 70 µl of this mixture were quickly pipetted on a precoated slide, which was placed on cooled trays. Trays were kept in the cooling chamber before usage. Each slide had space for two spots with a volume of 70 µl.
- Each slide was immediately covered with one cover slip (20x20 mm) and stayed in the cooling chamber for a minimum of 5 minutes before the cover slips got removed. During this time the agarose set.

Treatment with H₂O₂:

- After the cover slips have been removed from the slides, they were placed in a staining jar containing a cooled H₂O₂-solution (100 µM) and incubated for 5 minutes in a cooling chamber (4 °C).
- Then the slides were washed 3 times in PBS to remove H₂O₂.

Lysis:

- The lysis solution (lysis solution with 1 % triton X) was filled into jars.
- After H₂O₂ incubation and removing the cover slips from all other slides, they were placed in the prepared lysis solution for 75 minutes in the cooling chamber.
- Slides without H₂O₂ incubation were incubated separately from slides with H₂O₂ incubation.

Enzyme treatment:

- Enzyme reaction buffer was prepared.
- Slides for enzyme incubation (“buffer”, “FPG” and “ENDO III”) were washed in staining jars with enzyme reaction buffer (4 °C) 3 times for 5 minutes respectively.
- Enzyme dilutions (1:3000) were prepared.
- Buffer was drained off the slides.
- The agarose embedded cells (note: 2 gels/slide) were covered with 50 µl of either enzyme solution (FPG or ENDOIII) or enzyme reaction buffer (as negative control) and cover slips (22x22 mm). Slides were placed in a moist box and incubated in a drying chamber at 37 °C for 30 minutes.
- Meanwhile all other slides (H₂O₂ and “lysis”) stayed in the lysis solution.

Alkaline treatment and electrophoresis: (see Figure 13)

- All cover slips were removed before they were placed in the electrophoresis.
- The electrophoresis tank was placed in the cooling chamber at 4 °C.
- Slides were placed on the platform in the electrophoresis tank with the gel upwards and the labelling areas towards the same direction. Incomplete rows were filled up with precoated but blank slides. (In every electrophoresis 42 microscope slides had place that means 5 samples and one control sample.)
- Slides were incubated in alkaline electrophoresis buffer for 20 minutes. (unwinding phase)
- After the alkaline treatment, the electrophoresis was performed for 30 minutes at around 300 mA, 25 V and 8 W. The current was adjusted by adding (if too low) or removing (if too high) alkaline electrophoresis buffer at a switched off power supply.

Neutralisation:

- Slides were taken out from the electrophoresis and neutralized by rinsing 5 minutes in cold neutralization buffer and another 5 minutes in double distilled water at 4 °C.
- Finally the slides were dried at room temperature overnight in darkness and stored until staining.

Staining:

- 30 μ l of the ethidium bromide solution (20 μ g/ml) was placed on the slide and covered with a coverslip (22x22 mm).
- For examination two out of three slides were selected and at any time 50 cells per gel were counted. In case values of the two slides differed too much the third one was additionally determined.

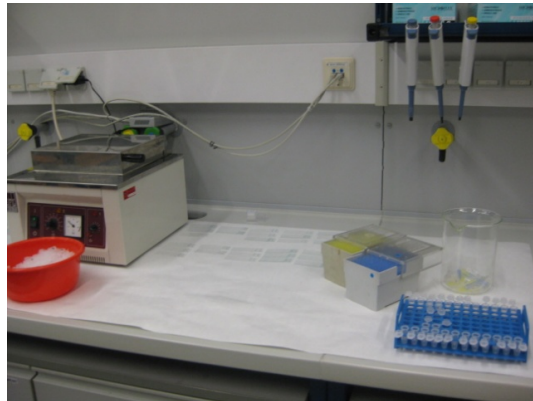
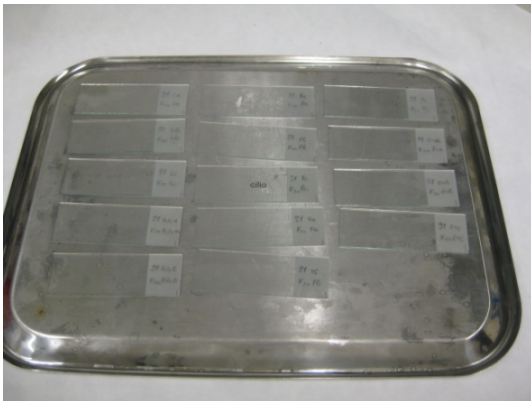


Figure 12: Slides on the tray before start of embedding cells in agarose.

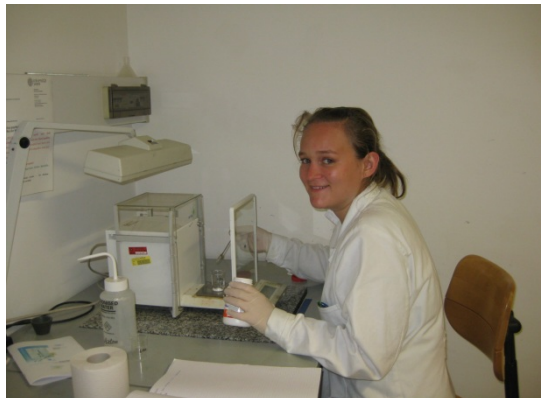
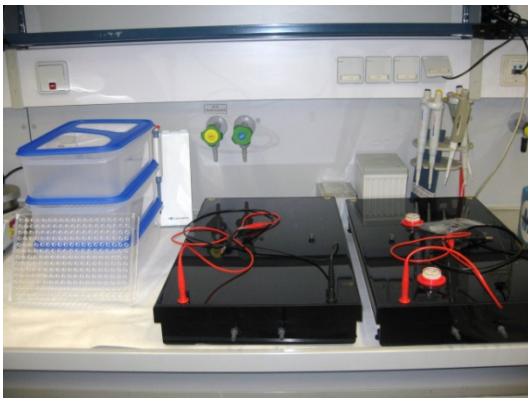


Figure 13: Electrophoresis (left) and lab work (right)

3.4. Statistical Analysis

Statistical analysis of all the data was performed with the program "Statistical Program for Social Sciences (SPSS) version 17.0 for Windows,". At first, the boxplot was used in order to identify extreme values. All the data were proved for normal distribution using the 1-sample Kolmogorow-Smirnow-Test (KS-Test). For two- group Comparison of genomic damage (e.g. Gilbert and Control) the "Independent sample t-Test" was used as all data were normally distributed. The ANOVA-test (Tukey- HSD) was used for three- or more-group Comparison (e.g. Bilirubin tertiles). Correlations between variables were tested using the bivariate "Pearson Correlation". Finally a stepwise linear regression analysis was performed.

All results are expressed as mean values $\pm$ standard deviation (SD). Differences with p value < 0.05 were considered as statistically significant.

The subjects were divided in 2 groups according to the level of unconjugated bilirubin. The groups were respectively "Gilbert's Syndrome" (UCB levels >1 mg/dl; $[17, 1 \mu\text{mol/l}]$), "Control" (UCB levels <1 mg/dl; $[17, 1 \mu\text{mol/l}]$). Additionally a third group 'not defineable' was formed, which included subjects that could not be allocated to either one of the groups because bilirubin levels were close to 1 mg/dl. When DNA damage in Gilbert's syndrome and control group was compared, data of the 'not defineable group' were excluded from statistical analysis. Furthermore DNA damage was compared after dividing the study population into three similar groups of bilirubin levels; group 1 (low bilirubin levels $(0.24- 0.65 \text{ mg/dl})[4.1-11.1 \mu\text{mol/l}]$, group 2 (moderate bilirubin: $0.66 -1.21 \text{ mg/dl } [11.3-20.7 \mu\text{mol/l}]$ and group 3 (high bilirubin: $1.22 -4.3 \text{ mg/dl } [20.9-73.5 \mu\text{mol/l}]$ (with 'not defineable' included $n = 16$).

Moreover, the data was stratified for age and gender. The two age groups were respectively 'young' (test persons < 30 years) and 'older' (test persons ≥ 30 years).

In this work obtained results for DNA single- and double-strand breaks detected by lysis in Gilbert's syndrome and control are discussed. Moreover oxidative DNA damage in terms of FPG- sensitive sites and ENDO III- sensitive sites are demonstrated and discussed in detail. Results for lysis and resistance towards oxidative damage by oxidative challenge with H_2O_2 are demonstrated and discussed in the work of Barbara Rittmannsberger "Evaluation of DNA Damage in Gilbert's Syndrome Subjects in H_2O_2 -treated and untreated PBMCS".

4. Results and discussion

The framework of this work aimed at investigating the relationship between bilirubin level and DNA damage. Moreover, it should be explored whether individuals with Gilbert's syndrome and characteristically increased bilirubin concentrations show improved resistance towards DNA damage in comparison to controls.

The study population consisted of 103 subjects. Later on 3 subjects were excluded from the study, because one subject turned out to be a smoker, one showed abnormal blood glucose levels and one had a BMI > 35. The descriptive statistic of the study population can be found in Table 3.

Table 3: Description of the study population

	Gilbert	Control	Not defineable
Number	45	39	16
Men	35	23	12
Women	10	16	4
Age (years)	33 ± 13	32 ± 12	26 ± 6
UCB (mg/dl)	1.9 ± 0.83	0.5 ± 0.19	0.7 ± 0.21

Data are means ± SD. UCB: Serum concentration of unconjugated bilirubin.

4.1. DNA damage

Data of the comet assay were statistically analysed for 98 subjects from a total of 100; 2 values were excluded from further analysis because they showed extremely high values for DNA damage and hardly any cells were on the microscopic slide.

Mean DNA damage expressed as % DNA in tail in the whole study population for lysis was 5.1 ± 1.23 ; for FPG– sensitive sites (oxidised purines) 4.9 ± 2.77 and for ENDO III– sensitive sites (oxidised pyrimidines) 2.2 ± 1.25 .

4.1.1. DNA damage in Gilbert's Syndrome and Control

Subjects were divided in 2 groups according to the level of unconjugated bilirubin which was detected by HPLC (further details can be obtained from the diploma thesis of Katharina Marisch "The DNA protecting effect of unconjugated bilirubin"). The groups were respectively "Gilbert's Syndrome" (UCB levels >1 mg/dl; [17, 1 μ mol/l]), "Control" (UCB levels <1 mg/dl; [17, 1 μ mol/l]). Table 4 summarises the results of DNA damage in Gilbert and control.

Table 4: DNA damage in Gilbert's syndrome and Control

	Gilbert's Syndrome	Control	pValue
Number	45	37	
% DNA in tail Lysis	5.3 $\pm$ 1.35	5.0 $\pm$ 1.18	0.252
% DNA in tail FPG	5.3 $\pm$ 3.13	4.6 $\pm$ 2.32	0.231
% DNA in tail ENDO III	2.1 $\pm$ 1.17	2.5 $\pm$ 1.38	0.245

There were no significant differences in mean DNA damage in Gilbert and control group in strand breaks and FPG- or ENDO III- sensitive sites. The mean values for single- and double-strand breaks (lysis) in Gilbert were slightly higher (6 %) compared to the control group. FPG- sensitive sites were 15.2 % higher in the Gilbert group. Values of ENDO III- sensitive sites were 16 % lower in the Gilbert group in comparison to the control group but not significantly.

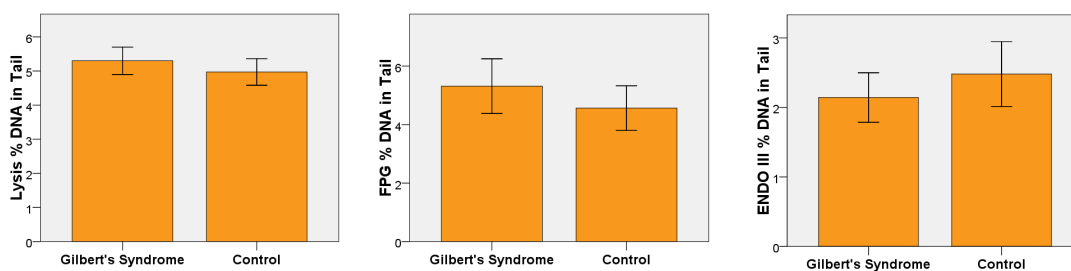


Figure 14: DNA damage in Gilbert's syndrome and Control

We expected lower DNA damage in the Gilbert group due to elevated bilirubin levels and its strong proposed antioxidative potential. This is the first study which investigated the effect of elevated bilirubin towards genotoxicity using the comet assay. Thus, the results cannot be compared with existing literature. In order to detect the protective effect of bilirubin at DNA level further research is needed.

Throughout the whole study population, higher mean values of % DNA in tail for FPG- sensitive (4.9 ± 1.23) sites compared to Endo III- sensitive sites (2.2 ± 1.25) were found. This is comparable to other study results [BOTTO et al., 2002; MOSER et al., 2011].

An explanation for higher FPG values is that, in the comet assay, the FPG protein detects 8-oxo-dG (8-Oxo-7, 8-dihydro-2'-deoxyguanosine) which is easily formed and the most frequent DNA lesion [MOLLER and LOFT, 2006]. Besides oxidative damaged purines, FPG also detects alkylation damage especially N-7 alkylation of guanine with high sensitivity. There are low background levels of alkylated bases in normal human lymphocytes caused by various endogenous mechanisms or by exogenous alkylating substances. Thus, increased values of FPG cannot be directly linked to increased presence of oxidative damage. ENDO III on the contrary detects solely oxidised pyrimidines and is more specific for oxidative DNA damage [SPEIT 2004; COLLINS et al., 2001; DUSINSKA and COLLINS 2008].

There is a relatively high intra- and interindividual variation in the basic level of DNA damage among healthy individuals which can be explained by the individual and interlaboratory factors [DUSINSKA and COLLINS 2008].

Some authors also indicate that a state of oxidative stress is required to observe the antioxidant properties of compounds more effectively. As most subjects were relatively young and all non-smokers (up to 5 cigarettes) it can be assumed, that level of oxidative stress in the subjects was low.

4.1.2. DNA damage in bilirubin tertiles

Furthermore the total study population was divided into three similar groups according to their serum concentration of unconjugated bilirubin (bilirubin tertiles). This was done to detect the impact of differing bilirubin levels on DNA damage more precisely. The mean values for DNA damage in the three groups of bilirubin are demonstrated in Table 5.

Table 5: DNA damage and bilirubin tertiles

	Tertile 1: 0.24-0.65 mg/dl	Tertile 2: 0.66 -1.21 mg/dl	Tertile 3: 1.22 -4.3 mg/dl	pValue
Number	31	34	33	
% DNA in tail Lysis	4.9 ± 1.21	5.3 ± 1.14	5.1 ± 1.33	0.328
% DNA in tail FPG	4.7 ± 2.43	5.1 ± 3.08	4.8 ± 2.81	0.833
% DNA in tail ENDO III	2.3 ± 1.29	1.9 ± 1.32	2.2 ± 1.12	0.410

There were no significant differences between the 3 different bilirubin levels and DNA damage (see Figure 15).

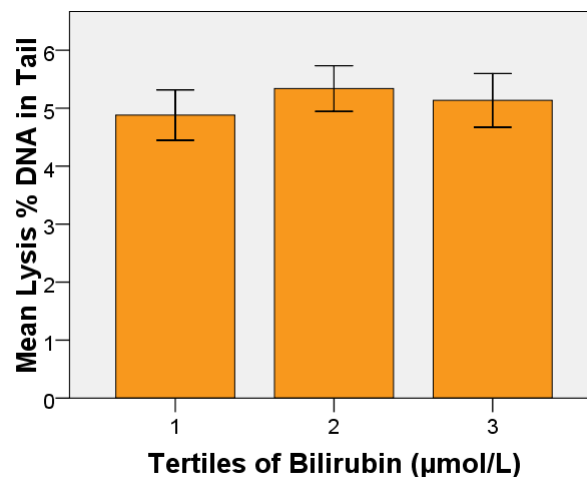


Figure 15: DNA damage in bilirubin tertiles (lysis)

4.1.3. Influencing factors on oxidative DNA damage

4.1.3.1. Age

In order to evaluate the impact of age on the level of oxidative DNA damage the subjects were allocated to two different age groups; young subjects < 30 years and old subjects $\geq$ 30 years. Results for mean DNA damage in Gilbert and control after stratification for age are summarised in Table 6.

Table 6: DNA damage in Gilbert and Control in two age groups (< 30 years; $\geq$ 30years)

		Gilbert's Syndrome	Control	pValue
< 30 years	Number	26	23	
	% DNA in tail Lysis	5.2 $\pm$ 1.35	4.8 $\pm$ 1.14	0.217
	% DNA in tail FPG	5.9 $\pm$ 3.74	5.1 $\pm$ 2.33	0.353
	% DNA in tail ENDO III	2.2 $\pm$ 1.07	2.5 $\pm$ 1.35	0.360
$\geq$ 30 years	Number	19	13	
	% DNA in tail Lysis	5.4 $\pm$ 1.37	5.3 $\pm$ 1.21	0.831
	% DNA in tail FPG	4.5 $\pm$ 1.84	3.7 $\pm$ 2.14	0.267
	% DNA in tail ENDO III	2.1 $\pm$ 1.31	2.4 $\pm$ 1.49	0.540

There were no significant differences in mean DNA damage in Gilbert's syndrome and control after the stratification for age.

Mean values of strand breaks and FPG- sensitive sites were slightly but insignificantly elevated in the Gilbert group in comparison to the control group. In younger subjects < 30 years, subjects with Gilbert's syndrome showed higher DNA damage for lysis (8.3 %) and for FPG- sensitive sites (15.7 %). In old subjects > 30 years, Gilbert subjects showed higher mean values for lysis (1.9 %) and values of FPG sensitive sites (21.6 %). On the contrary mean ENDO III- sensitive sites were lower in Gilbert's syndrome in both age groups < 30 years (12 %) and in subjects' $\geq$ 30 years (12.5 %) See Figure 16.

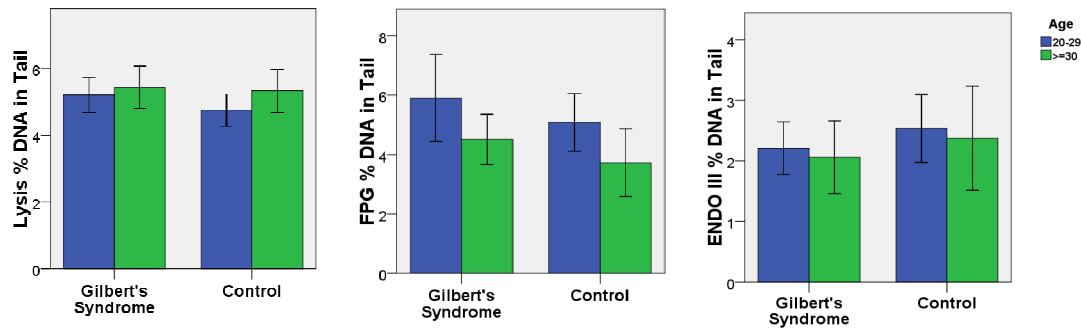


Figure 16: DNA damage in Gilbert's syndrome and Control and age

In Table 7 results for the impact of age on DNA damage in the whole study population are summarised.

Table 7: Impact of age on DNA damage in the whole study population

	< 30 years	≥ 30 years	pValue
Number	61	37	
% DNA in tail Lysis	5.0 ± 1.20	5.3 ± 1.28	0.233
% DNA in tail FPG	5.2 ± 3.10	4.3 ± 2.16	0.076
% DNA in tail ENDO III	2.2 ± 1.23	2.1 ± 1.29	0.906

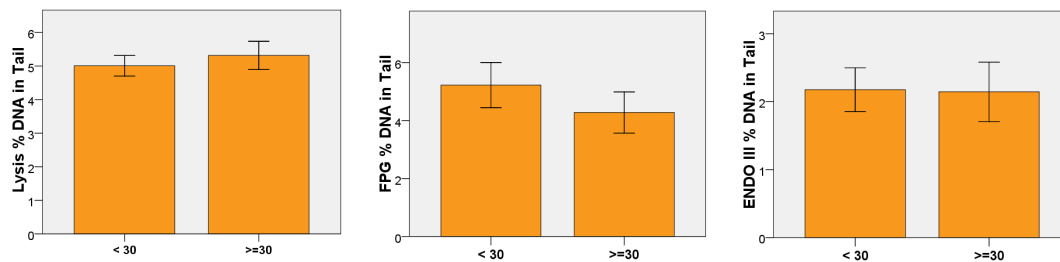


Figure 17: Impact of age on DNA damage in the whole study population

For all test persons splitted in two age groups, the results show that older subjects had slightly higher mean DNA damage (lysis) compared to younger people. However, the statistical significance was lacking. In the whole study population mean in strand breaks was 5.7 % lower in subjects < 30 years in comparison with people ≥ 30 years.

Concerning FPG- and ENDO III- sensitive sites, the opposite was observed. Values for FPG- sensitive were 20.9 % higher in subjects < 30 years and ENDO III was 4.7 % higher in subjects < 30 years.

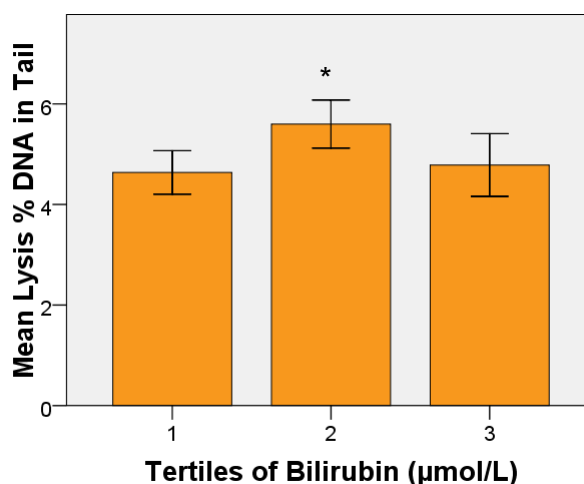


Figure 18: DNA damage and bilirubin tertiles in subjects < 30 years (lysis), * $p < 0.05$

After dividing the study population into 3 groups of bilirubin levels, a significant difference in the mean values of strand breaks ($p = 0.016$) was observed in subjects < 30 years (see Figure 18). Thus subjects with moderate levels of bilirubin 0.66- 1.21 mg/dl had significantly higher mean values of DNA damage in comparison with subjects with low bilirubin levels 0.24- 0.65 mg/dl.

There are only few studies which explored the effect of age on DNA damage measured by the comet assay. The studies concerning age and basal DNA damage, failed to show a clear effect of age and the results are still inconsistent [MOLLER et al., 2000].

Humphreys et al. aimed to investigate the effect of age on oxidative DNA damage using the alkaline comet assay and included FPG enzymes. The subjects were divided into three age groups (group 1: 20-35 years, group 2: 63-70 years and group 3: 75-82 years). The two older groups showed significantly decreased DNA damage in terms of H_2O_2 - induced strand breaks compared to the younger group. The rate of oxidised purines was significantly higher in the

oldest group in comparison with the two younger groups [HUMPHREYS et al., 2007].

In 2006 Dusinska et al., used the comet assay with FPG and detected a highly significant positive correlation between FPG- sensitive sites as well as between ENDO III- sensitive sites and age. The study included 239 Slovakian workers exposed to asbestos and 139 unexposed control subjects. In males single strand breaks increased with age but not in females. The subjects were between 21 and 88 years old, majority of the subjects were in the middle of the range [DUSINSKA et al., 2006].

In the study of King and co-workers DNA damage in four different age groups (Group 1: 35-39 years, Group 2: 50-54 years, Group 3: 65-69 years and Group4: 75-80 years) was measured. Results showed that there was no increase in mean DNA damage between groups 1, 2 and 4. Interestingly study subjects of group 3 showed highest level of DNA damage [KING et al., 1997].

Krajcovicová-Kudlácková and co-workers investigated DNA damage in relation to diet and age in 161 non- smoking women. DNA damage (DNA strand breaks, oxidised purines or oxidised pyrimidines) was measured in young women 20-30 years (46 vegetarians, 48 non-vegetarians) and older women 60-70 years (33 vegetarians, 34 non-vegetarians). In non-vegetarian women older subjects had significantly higher values of oxidised bases compared to younger subjects. In vegetarians, values were age-independent [KRAJCOVICOVÁ-KUDLÁCKOVÁ et al., 2008].

Further, the study of Kazimírová and co-workers showed significant positive correlation between age and oxidative DNA damage (net FPG- sensitive sites) in healthy non-vegetarian (average age 40 ± 1.7 years, ranging from 20 to 62). In vegetarians (average age 41 ± 2.0 years ranging from 20 to 69), there was a trend towards a negative association between age and FPG- sensitive sites [KAZIMÍROVÁ et al., 2006].

Nevertheless there are numerous studies which failed to show a correlation between DNA damage and age. For example in the study of Betti et al., no relation between age and DNA damage was found. The amount of strand breaks in DNA was measured in human lymphocytes from 200 healthy subjects using the comet assay [BETTI et al., 1995]. Another study also failed to show a significance impact of age on DNA damage in 160 elderly subjects (group 1: 60-69 years and group 2: > 70 years). Younger subjects tended to show lower values of strand breaks in comparison to older subjects although lacking statistical significance [Mendoza-Núñez et al., 2001]. Furthermore in a very recent study including 60 healthy male and 30 healthy females (25-35 years), there was no significant difference concerning age and basal DNA damage measured with the comet assay [DIXIT, 2011].

Aging is often accompanied by increased oxidative stress, due to higher rates of free radicals (ROS), a declined antioxidative system, reduced DNA repair and consequently increased rates of damage of important biomolecules. Throughout aging, increased rates of ROS are produced in the body resulting mainly from leakage of ROS of aged mitochondria [HUMPHREY et al., 2007].

In the current study FPG- sensitive sites were decreased in subjects ≥ 30 years without any statistical significance. It is possible that increased release of ROS during aging simultaneously stimulates DNA repair and also antioxidative defence in the body in order to protect the body against genomic damage accompanied by ageing [HUMPHREY et al., 2007].

In the study of Humphrey it was shown that the rate of DNA repair of 8-oxo guanine increases with age [HUMPHREY et al., 2007].

An important note has to be made on the study population. Subjects were divided into two age groups and all the subjects over the age of 30 were allocated to the older group. Most of the people in the study were around the age of 30 and only very few subjects were over 60 years (see Figure 19). The group of old subjects would have become too small to reach any statistical

power. 30 years cannot be considered as old and it can be assumed that a person aged 30 has a proper antioxidative defence system which copes effectively with the attack of free radicals. The small number of older subjects may be the reason why there were no significant differences in mean DNA damage in age. Further investigations in older people are necessary to investigate the impact of age on DNA damage. For getting more statistical power, it would be important to extend the number of subjects.

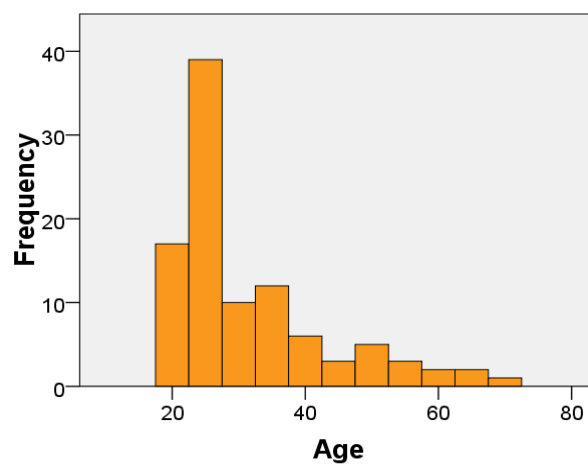


Figure 19: Distribution of age in the study population

4.1.3.2. Gender

The level of DNA damage in Gilbert and control were also compared after stratification of data in men and women. The results for the impact of gender on DNA damage in Gilbert's syndrome and control are summarised in Table 8.

Table 8: DNA damage in Gilbert and control and gender

		Gilbert's Syndrome	Control	pValue
Men	Number	35	22	
	% DNA in tail Lysis	5.5 ± 1.27	5.1 ± 1.23	0.245
	% DNA in tail FPG	5.4 ± 3.28	5.0 ± 2.46	0.588
	% DNA in tail ENDO III	2.2 ± 1.05	2.7 ± 1.38	0.157
Women	Number	10	15	
	% DNA in tail Lysis	4.5 ± 1.35	4.7 ± 1.12	0.626
	% DNA in tail FPG	4.9 ± 2.65	3.9 ± 2.03	0.326
	% DNA in tail ENDO III	2.0 ± 1.54	2.2 ± 1.38	0.749

No significant differences were observed in mean DNA damage when comparing Gilbert with control group after splitting data in men and women. Mean values of single- and double-strand breaks and FPG- sensitive sites were slightly, non-significantly higher in subjects with Gilbert's syndrome among both sexes. ENDO III- sensitive sites were lower in the Gilbert group in men and women (see Figure 20).

The mean in strand breaks, FPG- and ENDO III- sensitive sites was generally lower in women compared to men in both groups Gilbert and controls (see also Figure 20).

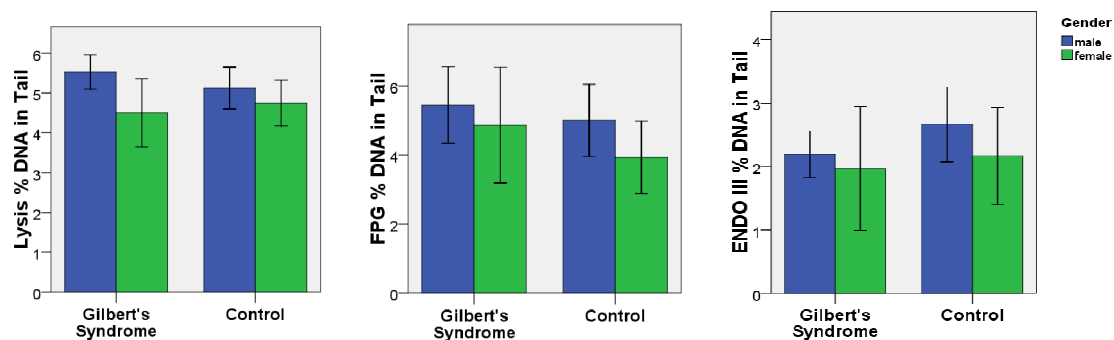


Figure 20: DNA damage in Gilbert's Syndrome and control and gender

The results summarised in Table 9 demonstrate the impact of gender on DNA damage in the whole study population.

Table 9: Impact of gender on DNA damage in the whole study population

	Male	Female	pValue
Number	69	29	
% DNA in tail Lysis	5.3 ± 1.21	4.6 ± 1.14	0.005
% DNA in tail FPG	5.1 ± 2.94	4.4 ± 2.31	0.283
% DNA in tail ENDO III	2.2 ± 1.21	2.0 ± 1.35	0.494

Women had significantly lower strand breaks in comparison to men. Women showed 15.2 % ($p < 0.01$) lower mean DNA damage for lysis, 15.9 % lower FPG- sensitive sites and 10 % lower mean ENDO III- sensitive sites (see Figure 21).

Higher levels of strand breaks in men were possibly caused by lower mean levels of vitamin B₁₂ (cobalamin) in men which showed protective effect towards DNA damage (see chapter 4.1.3.6). Furthermore men had higher mean levels of uric acid which showed a correlation with single- and double-strand breaks (see chapter 4.1.3.5).

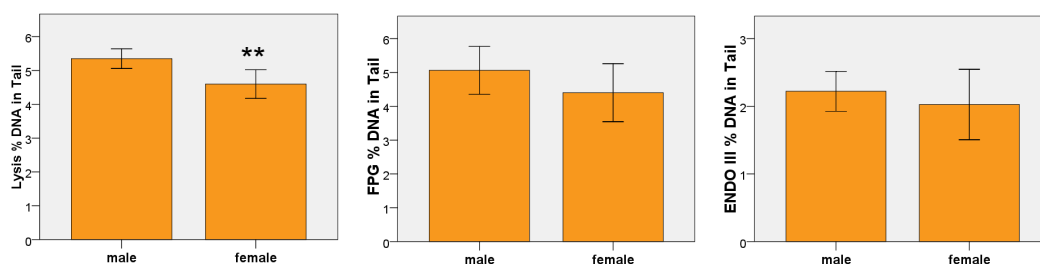


Figure 21: Impact of gender on DNA damage in the whole study population, ** $p < 0.01$

The difference in background levels of DNA damage between men and women is not clear yet. Studies which evaluated the difference between both sexes show conflicting results.

The results of the current study are in agreement with the result of a large cross-sectional study which reported that smoking increases DNA damage and that this effect is more significant in men compared to women [BETTI et al., 1994]. In the following year Betti studied DNA damage in an extended study population and it was shown that mean comet length in males was higher compared to women at borderline ($p = 0.053$) [BETTI et al., 1995].

Mendoza-Núñez et al., showed that elderly male had a 2-fold higher probability of having DNA damage compared to female [MENDOZA-NÚÑEZ et al., 2001].

In the study of Bajpayee et al., basic DNA damage in a young (age: 20-30) and healthy Indian male and female population illustrated that men had statistical significant higher level of DNA damage after lysis in comparison to females (Males % DNA in tail: 10.2 ± 2.96 ; Females % DNA in tail: 9.40 ± 2.83) [BAJPAYEE et al., 2002].

A very recent study with healthy individuals showed no significant difference among gender and strand breaks [DIXIT 2011]. Kazimírová who compared genomic damage in healthy non-vegetarians and vegetarians reported no sex difference in levels of DNA damage (strand breaks, FPG- and ENDO III-sensitive sites) [KAZIMÍROVÁ et al., 2006].

4.1.3.3. Albumin

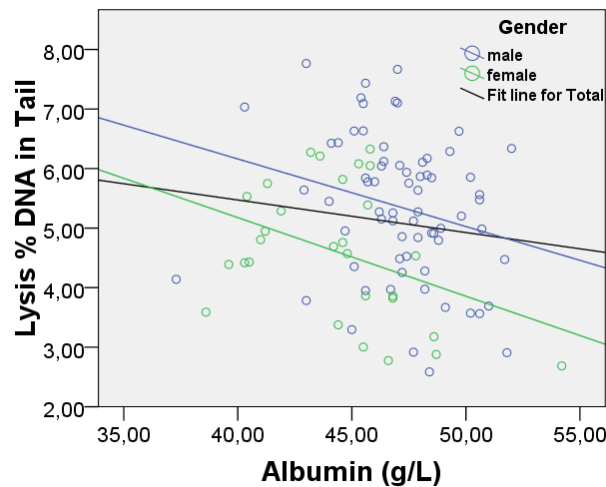


Figure 22: Correlation between Albumin and DNA damage (lysis)

Albumin correlated with single- and double-strand breaks in the whole study population although the correlation was not significant (see Figure 22).

A highly significant correlation between the concentration of albumin and DNA damage detected by lysis was observed in women < 30 years ($r = -0.565$; $p = 0.018$).

Albumin showed no correlation with oxidised DNA bases (FPG- and ENDO III-sensitive sites).

The major physiological function of albumin is the transport of lipid soluble substances such as fatty acids, hormones, cholesterol, bile pigments, drugs and metals [CANDIANO et al., 2009; ROCHE et al., 2008].

While several endogenous antioxidative systems are mainly active within the intracellular environment, albumin is considered as the most abundant protein in human plasma providing a major part of total antioxidative properties in the extracellular space [CANDIANO et al., 2009; ROCHE et al., 2008]. Low albumin levels are an important risk factor for all-cause and vascular mortality especially in men [GRIMM et al., 2009]. The strong antioxidant character of albumin can

possibly be explained by its ligand binding capacities which can assume different conformation and thus adapt the form of many different ligands [ROCHE et al., 2008].

Free transition metals Cu (II) and Fe (II) are involved in the generation of the highly reactive hydroxyl radical $\text{OH}^{\bullet}$ - via the Fenton's reaction from H_2O_2 .

Albumin binds copper tightly and iron with low affinity. It was shown that the addition of albumin to a H_2O_2 / Fe^{2+} / EDTA-catalysed oxidation system is able to reduce the consumption of H_2O_2 and thus the formation of hydroxyl radical, which at the same time decelerates free radical attack of other biomolecules [KOCHA et al., 1997]. It was shown by the same study that copper bound to albumin can still lead to the formation of $\text{OH}^{\bullet}$ - [KOCHA et al., 1997]. Although it is suggested that formed $\text{OH}^{\bullet}$ - are mostly directed to albumin, preventing more important biomolecules from oxidative damage. Additionally, metals bound to albumin are less susceptible to take part in the Fenton reaction [ROCHE et al., 2008].

4.1.3.4. Creatinine

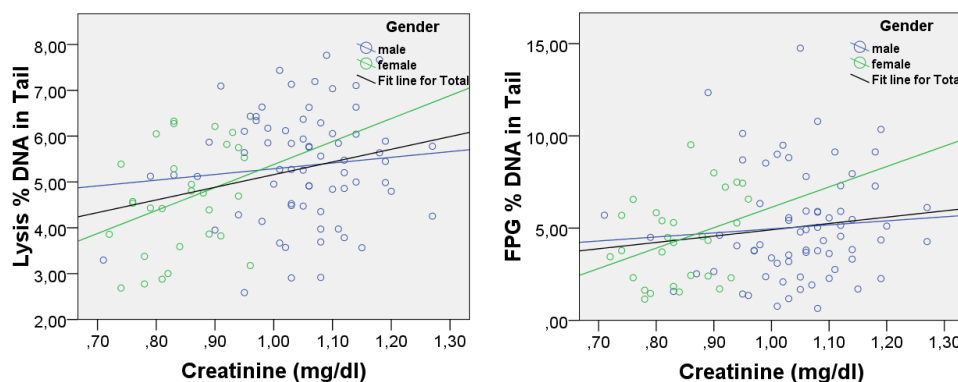


Figure 23: Correlation between Creatinine and DNA damage

Significant correlations were observed between creatinine levels and strand breaks detected by lysis in the whole study population ($r= 0.298$; $p= 0.003$) (see Figure 23).

This correlation was even stronger in younger subjects < 30 years ($r = 0.362$; $p = 0.004$). In women there was also a weak correlation although not significant ($r = 0.308$; $p = 0.104$).

Creatinine levels and FPG-sensitive sites also correlated non-significantly in women ($r = 0.336$; $p = 0.075$)

There was no correlation found between creatinine concentration and ENDO III-sensitive sites.

The results are in agreement with the study of Stoyanova et al., who measured the background level of genetic damage in 253 patients with chronic renal failure (CRF). In this study a positive correlation between creatinine levels and FPG-sensitive sites was found [STOYANOVA et al., 2010].

Stopper and co-workers also measured the impact of chronic renal failure on oxidative DNA damage and found that serum creatinine levels over 6 mg/dl led to an increase in oxidative DNA damage [STOPPER et al., 2001].

Baseline serum creatinine levels were shown to be related to increased risk of prostate cancer [WEINSTEIN et al., 2009]. Creatinine is an indicator for renal function and glomerular filtration. Thus, diminished renal function or renal disease leads to increased creatinine levels [WEINSTEIN et al., 2009].

The mean concentrations of creatinine in the study population were 0.98 mg/dl which is in the normal range (0.6-1.36 mg/dl). Due to decreased muscle mass, women generally have lower concentrations of creatinine [DORMANN et al., 2009].

Higher levels of creatinine showed correspondence with increased DNA damage in women indicating that increased creatinine concentrations might have negative impact on DNA stability.

4.1.3.5. Uric acid

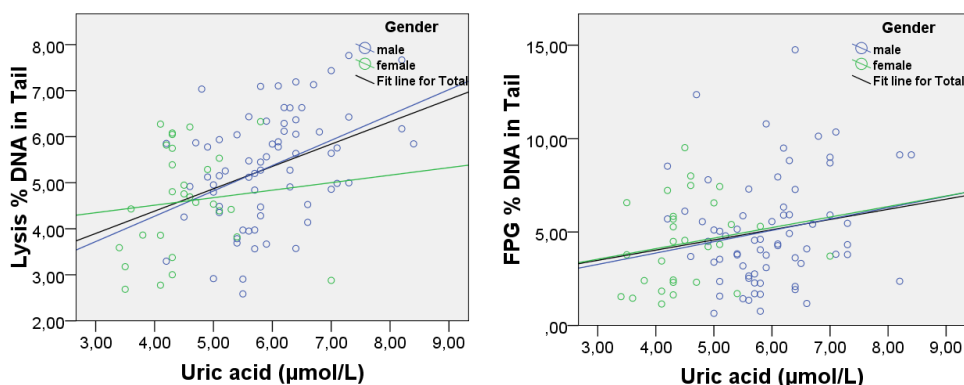


Figure 24: Correlation between Uric acid and DNA damage

A highly significant and strong correlation was observed between Uric acid levels and DNA damage (lysis) in the whole study population ($r = 0.426$; $p = 0.000$) (see Figure 24).

This correlation was highly significant in men ($r = 0.411$; $p = 0.000$) and men < 30 years ($r = 0.444$; $p = 0.003$).

The strongest correlation was observed between Uric acid levels and lysis in young subjects < 30 years ($r = 0.533$; $p = 0.000$).

Another correlation, weak but significant, was found between Uric acid and FPG- sensitive sites in the whole study population ($r = 0.213$; $p = 0.035$).

There was no significant correlation found, between uric acid levels and ENDO III- sensitive sites.

Uric acid (UA) is produced by the breakdown of purine nucleotides, which are principal constituents of cellular energy stores ATP and also DNA and RNA. In most mammals, urate is further metabolised to allantoin with the aid of Uricase which leads to substantially reduced UA levels in plasma [RUGGIERO et al., 2006]. In humans the enzyme Uricase is not expressed leading to increased levels of UA. Ames suggested, that elevated levels of UA can protect humans from aging and cancer due to its strong antioxidant potential [AMES et al.,

1981]. Nowadays, it is still controversial whether high UA levels can suppress increased oxidative stress and oxidative stress related disease [ROGGIERO et al., 2006]. Several studies indicate that high levels of UA are a risk factor for increased cardiovascular and all-cause mortality [NISKANEN et al., 2004] and also a risk factor for cancer [KOLONEL et al., 1994]. It was suggested that there is a positive relation between uric acid and pro inflammatory markers which are elevated in many chronic diseases especially in older people [RUGGIERO et al., 2006]. It was also found that cancer-patients show elevated levels of uric acid caused by malignancy, which is characterised by high proliferation rate of diseased tissue and increased nucleic acid turnover [STRASAK et al., 2008].

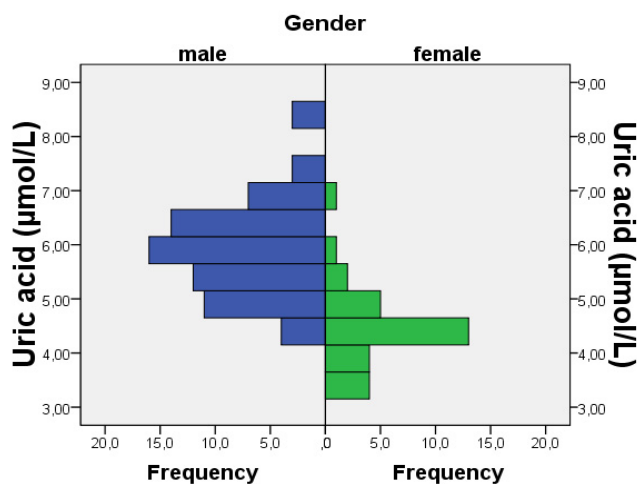


Figure 25: Distribution of uric acid levels in men and women

In the distribution plot it is clearly illustrated that men of the study group had higher ranges of uric acid levels ($\mu\text{mol/L}$) (5.9 ± 0.90) than females (4.5 ± 0.74). The values are consistent with the measurements of Choi and co-workers [CHOI et al., 2005]. Higher mean levels of uric acid in men might be an explanation for the increased levels of single- and double strand breaks in men compared to women (see chapter 4.1.3.2).

4.1.3.6. Vitamin B₁₂ (cobalamin)

Vitamin B₁₂ is known to play a crucial role in DNA metabolism. In order to examine whether vitamin B₁₂ has an impact on values of strand breaks and/or modified DNA bases, the study population was divided into three similar groups with different vitamin B₁₂ concentrations (see Table 10).

Table 10: DNA damage and Vitamin B₁₂ tertiles

	Tertile 1: 0.5- 1.4 pM/L	Tertile 2: 1.4- 2.0 pM/L	Tertile 3: 2.0- 4.0 pM/L	pValue
Number	37	24	35	
% DNA in tail Lysis	5.8 ± 0.80	5.0 ± 1.35	4.5 ± 1.20	0.000
% DNA in tail FPG	6.0 ± 3.18	5.3 ± 2.60	3.5 ± 1.74	0.000
% DNA in tail ENDO III	2.5 ± 1.46	2.0 ± 1.11	2.1 ± 1.10	0.284

There was a strong inverse relation between levels of Vitamin B₁₂ measured in erythrocytes and strand breaks and FPG- sensitive sites. Any increase in Vitamin B₁₂ concentration was followed by reduced mean levels of strand breaks and FPG- sensitive sites. Higher levels of Vitamin B₁₂ also reduced ENDO III- sensitive sites although lacking statistical significance.

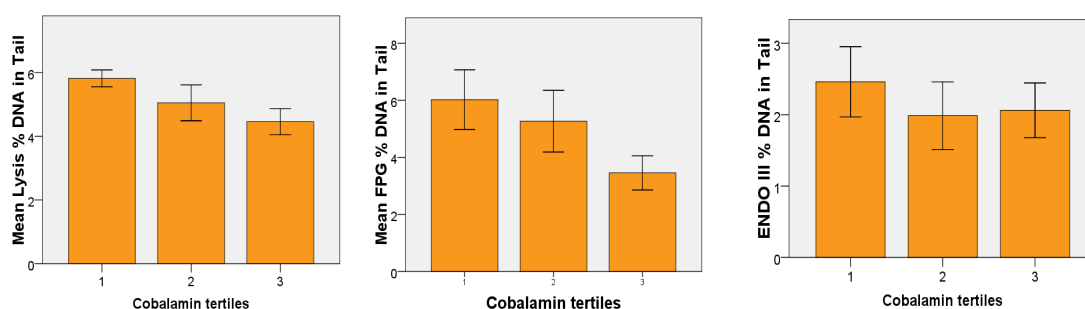


Figure 26: DNA damage and vitamin B₁₂ tertiles

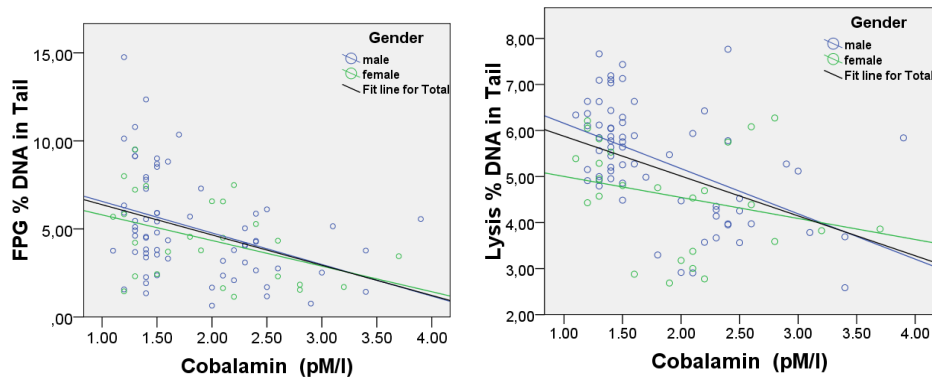


Figure 27: Correlation between Vitamin B₁₂ in Erythrocytes and DNA damage

Vitamin B₁₂ correlated strongly and highly significant with strand breaks (lysis) in the whole study population ($r = -0.452$; $p = 0.000$) and in men ($r = -0.511$; $p = 0.000$). The correlation was also observed in both age groups (< 30 years: $r = -0.464$; $p = 0.000$; > 30 years: $r = -0.420$; $p = 0.012$).

Moreover, a highly significant correlation between B₁₂ and FPG-sensitive sites in the whole study population was observed ($r = -0.397$; $p = 0.000$). This correlation was also observed in both genders (men: $r = -0.384$; $p = 0.001$; women: $r = -0.422$; $p = 0.032$) and in both age groups (< 30 years: $r = -0.436$; $p = 0.001$; > 30 years: $r = -0.391$; $p = 0.020$). A very strong and significant correlation was observed in women > 30 years ($r = -0.611$; $p = 0.046$).

There were no significant correlations observed between B₁₂ and ENDO III-sensitive sites.

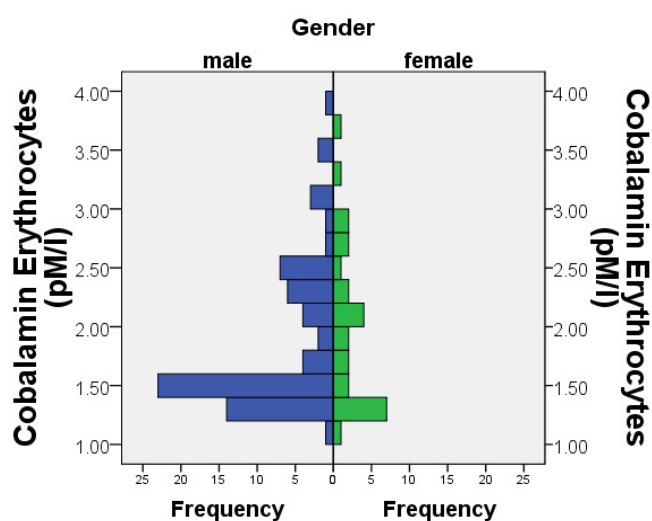


Figure 28: Distribution of erythrocyte (red blood cell) cobalamin in men and women

The mean level of cobalamin in red blood cells in the study population was 1.9 ± 0.65 pM/l. In men levels were slightly lower (1.8 ± 0.64) compared to women (1.9 ± 0.68). Lower levels of cobalamin in men may be an explanation for the higher values of DNA damage that was observed in men in comparison to women (see chapter 4.1.3.2).

Summarising the results, vitamin B₁₂ showed a protective effect towards DNA damage. Thus, in our study vitamin B₁₂ was able to minimise the formation of strand breaks but also oxidation of DNA bases especially purines.

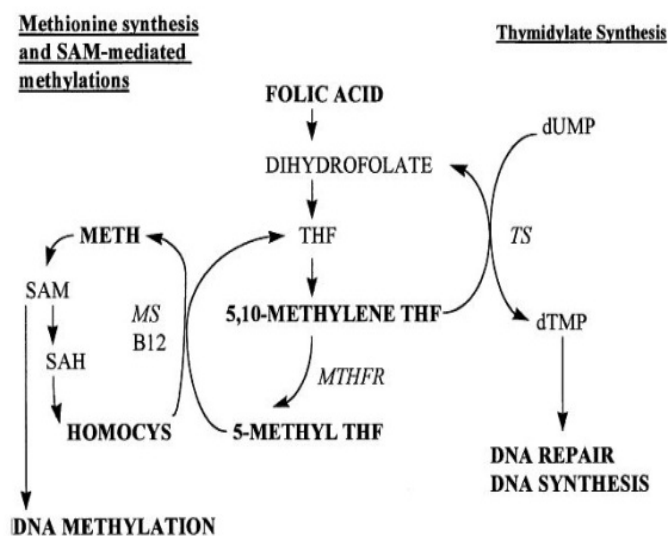


Figure 29: Pathway of folic acid and Vitamin B₁₂ [Fenech, 2001]

Together with folic acid, vitamin B₁₂ plays a crucial role in DNA metabolism and DNA repair. It is required for the synthesis of S-adenosyl methionine (SAM), which is the common methyl donor required for the maintenance of methylation patterns in DNA (see Figure 29). Vitamin B₁₂ is a water-soluble essential vitamin, thus mammals are unable to synthesise Vitamin B₁₂ de novo. The vitamin must be obtained from the diet or from microbial breakdown in the gut [DUTHIE, 1999].

Low concentrations of B₁₂ and folic acid lead to deficient methylation of uracil to thymidine and subsequent incorporation of uracil into DNA [FENECH, 2001].

Excessive incorporation of uracil into DNA might cause point mutation, single- and double-strand breaks, chromosome breakage and micronucleus formation [FENECH, 2001]. Both folate and B₁₂ deficiency independently or together have been shown to cause chromosome damage and micronucleus formation in vivo in humans [KAZIMÍROVÁ et al., 2006].

A strong negative correlation between concentrations of vitamin B₁₂ and oxidative DNA damage in peripheral mononucleated cells was found. Most

studies did not detect an effect of Vitamin B₁₂ level on markers of oxidative DNA damage.

Milic and co-workers failed to detect an effect of Vitamin B₁₂ and folic acid levels on DNA damage in terms of strand breaks and alkali labile sites [MILIC et al., 2010]. Also the study of Zijno et al., 2003 tail moment was examined by comet assay and was not affected neither by Vitamin B₁₂ nor folic acid [ZIJNO, 2003].

A more frequent lesion of DNA caused by folate or Vitamin B₁₂ deficiency is the uracil incorporation (UrMis) into DNA instead of thymidine. Thus, studies using a modified version of comet assay by inclusion of the enzyme uracil glycosylase show more clear results concerning association between the two essential vitamins and their role in DNA metabolism.

4.1.3.7. Folic acid

Additionally the study population was divided into three groups of different folic acid levels. Table 11 summarises mean values of DNA damage for red cell folate within the three groups.

Table 11: DNA damage and red cell folate tertiles

	Tertile 1: 38.90- 72.80 ng/mL	Tertile 2: 74.10- 152.40 ng/ mL	Tertile 3: 157.10- 510.40 ng/mL	pValue
Number	31	32	33	
% DNA in tail Lysis	5.1 ± 1.55	5.3 ± 1.01	5.0 ± 1.18	0.644
% DNA in tail FPG	4.3 ± 2.95	4.8 ± 2.13	5.8 ± 3.19	0.294
% DNA in tail ENDO III	1.9 ± 1.05	2.3 ± 1.34	2.4 ± 1.37	0.097

Results show that there was no significant difference in mean DNA damage between the three different folic acid concentrations.

FPG- and ENDO III- sensitive sites tended to increase with higher red cell folate concentration but differences in DNA damage were not significant.

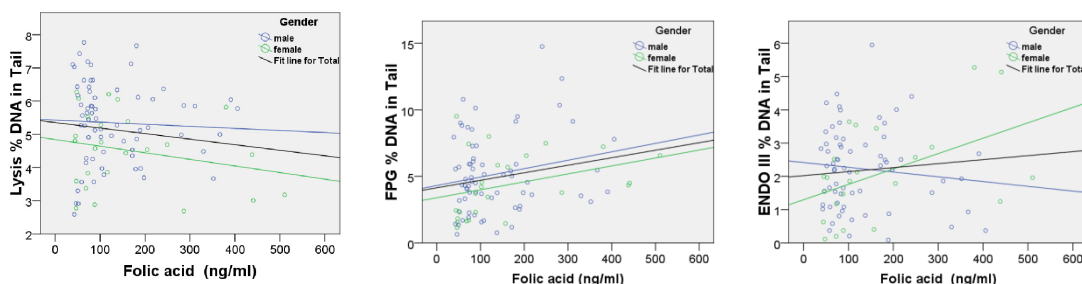


Figure 30: Correlation between red cell folate and DNA damage

Correlations were observed between folic acid in erythrocytes and DNA damage especially in women (see Figure 30).

In women ≥ 30 years a positive correlation between red cell folate and strand breaks ($r = 0.427$; $p = 0.219$) was observed but results were not significant. On the contrary in women < 30 years a significant negative correlation was found between red cell folate and strand breaks ($r = -0.524$; $p = 0.045$).

Correlations were found between folic acid and FPG- sensitive sites in women, however statistical significance was lacking ($r = 0.345$; $p = 0.091$). This correlation was even stronger and also significant in women ≥ 30 years ($r = 0.648$; $p = 0.043$).

Folic acid also correlated with ENDO III- sensitive sites in women generally ($r = 0.469$; $p = 0.024$) and strong correlation was found in women ≥ 30 years ($r = 0.793$; $p = 0.019$).

To sum up the results, folic acid mainly had impact on DNA damage in females. It was found that folic acid influenced especially oxidation damage in terms of FPG- and ENDO III- sensitive sites. With regard to strand breaks results are not clear; in women ≥ 30 years folic acid increased DNA damage whereas in women < 30 it reduced the amount of strand breaks in DNA.

In the study of Duthie and Hawdon folate supplementation did not show an effect on level of oxidised pyrimidines in vitro, thus suggesting that folate deficiency increases uracil miscorporation specifically and does not influence oxidative DNA damage [DUTHIE and HAWDON 1998].

On the other hand in a case-control study red cell folate did correlate negatively with FPG- sensitive sites [HUSSEIN et al., 2005]. Also Courtemanche and co-workers showed that folate deficiency leads to increased DNA damage in primary lymphocytes [COURTEMANCHE et al., 2004].

It was proposed by Duthie that DNA damage occurs at folic acid concentrations, which are routinely measured in plasma from the human population [DUTHIE and HAWDON 1998].

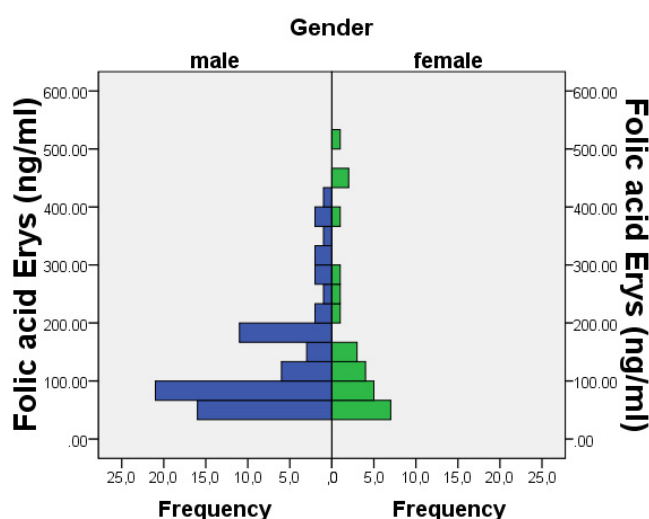


Figure 31: Distribution of red cell folate in men and women

The distribution plot shows that the study subjects had mean red cell folate concentrations of 139.8 ng/ml. In females, average folate concentrations were slightly higher 159.1 ± 139.33 in comparison to men 132.4 ± 93.02 .

The recommended daily intake of micronutrients may not be optimal for the purpose of protection from genomic damage. According to Fenech, higher intake of folic acid than RDI (recommended dietary intakes) is required for the

protection against DNA damage. In general, 464 ng/ml red cell folate concentrations were shown to correspond to minimisation of DNA damage [FENECH, 2001].

The average folate concentration in the study population was probably too low for minimisation of DNA damage. Therefore the increased strand breaks detected by lysis in women ≥ 30 years might be linked to folate concentration due to increased uracil misincorporation and nicks in the DNA which occur during the repair process.

4.1.3.8. Correlations within DNA damage endpoints

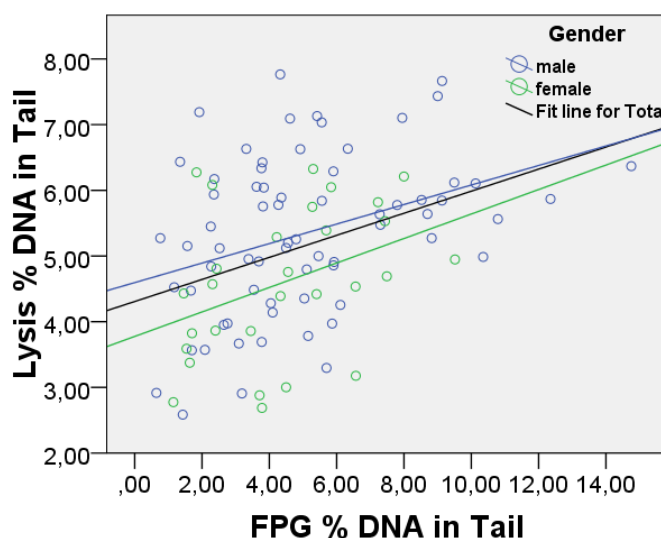


Figure 32: Correlation between DNA damage endpoints lysis and FPG- sensitive sites

There were strong correlations observed among the individual DNA damage endpoints. For instance lysis and FPG-sensitive sites, correlated highly significant in the whole study population ($r = 0.378$; $p = 0.000$), in men ($r = 0.376$; $p = 0.044$) and women ($r = 0.362$; $p = 0.002$) (see Figure 32). Lysis and FPG-sensitive sites also correlated in people < 30 years ($r = 0.382$; $p = 0.002$) and people ≥ 30 years ($r = 0.485$; $p = 0.002$). The results indicate that higher levels of strand breaks detected by lysis are accompanied by higher values of FPG-sensitive sites (oxidised purines). Up to now, no studies investigated the correlation between oxidised bases and strand breaks.

4.1.4. Linear regression

A stepwise linear regression model was made in order to estimate how much of DNA damage was explained by independent variables that were considered. Included variables were: Albumin, uric acid, creatinine, age, unconjugated serum bilirubin, vitamin C, glutathione, folic acid in erythrocytes, vitamin B12 in erythrocytes and BMI.

4.1.4.1. Lysis

Table 12: Linear regression model Lysis

Test subjects (n)	Predictors	Adjusted R ²	pValue
All test subjects (92)	Cobalamin	0.206	0.000
	Uric acid	0.332	0.000
	Albumin	0.387	0.004
	Glutathion	0.411	0.034
	Creatinine	0.434	0.035
Men (67)	Cobalamin	0.255	0.000
	Uric acid	0.370	0.000
	Glutathion	0.436	0.000
	Albumin	0.466	0.037
Women (25)	Albumin	0.142	0.036
	Vitamin C	0.261	0.041
< 30 years (58)	Uric acid	0.270	0.000
	Glutathion	0.425	0.000
	Cobalamin	0.495	0.005
	Vitamin C	0.540	0.016
	Albumin	0.594	0.006
	Creatinine	0.622	0.034
≥ 30 years (34)	Cobalamin	0.168	0.009
	BMI	0.257	0.036

Table 12 summarises the results for the stepwise linear regression model for lysis. In the whole study population 43.4% of DNA was explained by cobalamin, uric acid, albumin, glutathione and creatinine whereby cobalamin, uric acid and albumin already explained 38,7 % of the DNA damage.

In men DNA damage was mainly influenced by cobalamin, uric acid, glutathione and albumin which summed up to a total of 46.6 % of DNA damage.

In women albumin and vitamin C had the greatest impact on DNA damage explaining 26.1 % of DNA damage.

According the two age groups, in the younger subjects (< 30 years) DNA damage was mainly influenced by the variables uric acid, glutathione, cobalamin, vitamin C, albumin and creatinine which together explained 62.2 % of DNA damage. In older subjects ($\geq$ 30 years) cobalamin and BMI explained 25.7 % of DNA damage.

Summarising the results of the linear regression model it can be seen, that all together cobalamin, uric acid and albumin had the greatest impact on single- and double-strand breaks detected by lysis. As already discussed in earlier chapters cobalamin and albumin showed a correspondence with DNA damage and reduced it whereas uric acid rather increased the level of DNA damage.

4.1.4.2. FPG

Table 13: Linear regression model for FPG

Test subjects (n)	Predictors	Adjusted R ²	pValue
All test subjects (92)	Cobalamin	0.149	0.000
	Folic acid	0.179	0.043
Men (67)	Cobalamin	0.132	0.001
Women (25)	Cobalamin	0.156	0.029
	Creatinine	0.268	0.045
< 30 years (58)	Cobalamin	0.175	0.001
$\geq$ 30 years (34)	Cobalamin	0.145	0.015

In the whole study population the greatest impact on DNA damage in terms of FPG- sensitive sites was shown by cobalamin and folic acid which together explained 17.9% of DNA damage.

In men, FPG- sensitive sites were mainly influenced by cobalamin by 13.2% and in women cobalamin and creatinine had the greatest impact on DNA damage explaining 26.8% of total FPG- sensitive sites.

In the younger subjects (< 30 years) cobalamin explained 17.5% of oxidised purines and in older subjects ($\geq$ 30years) cobalamin explained 14.5% of DNA damage.

To sum up cobalamin showed the greatest effect on oxidised purines in the whole study population also after stratification for sex and age. The results from linear regression model indicate that cobalamin plays a crucial role when it comes to oxidative stress and DNA damage although the mechanism is still not known.

4.1.4.3. ENDO III

Table 14: Linear regression model for ENDO III

Test subjects (n)	Predictors	Adjusted R ²	pValue
All test subjects (85)	No model found	-	-
Men (65)	No model found	-	-
Women (23)	Folic acid	0.183	0.024
	Glutathione	0.366	0.015
	Vitamin C	0.501	0.020
< 30 years (56)	No model found	-	-
$\geq$ 30 years (32)	No model found	-	-

There were only models found explaining DNA damage in women. In women 50.1% of ENDO III- sensitive sites were explained by the variables folic acid, glutathione and vitamin C.

These results indicate that folic acid but also glutathione and vitamin C have an impact on DNA damage in terms of oxidised pyrimidines in women.

5. Conclusion

The framework of the present work aimed at exploring the antigenotoxic effect of bilirubin. Furthermore it was evaluated whether subjects with Gilbert's syndrome (mild hyperbilirubnemia) are better protected against oxidative stress and oxidative stress mediated DNA damage.

In order to assess the antigenotoxic properties of bilirubin the amount of DNA damage in peripheral blood mononucleated cells (PBMCs) in subjects with Gilbert's syndrome and control was analysed. As a method the comet assay was conducted which allows the measurement strand breaks in DNA efficiently. Moreover, oxidative modified DNA bases were analysed by inclusion of lesion-specific enzymes; which give more specific information about oxidative stress related DNA damage.

Due to the strong reported antioxidative capacities of bilirubin an inverse relation between bilirubin levels and DNA damage was expected. In fact the hypothesis could not be confirmed, as there were no significant differences in DNA damage between GS and control. Furthermore, there were no differences in DNA damage after subdividing the study population in 3 similar groups of different bilirubin levels (bilirubin tertiles).

However the experiment delivered other findings. Various endogenous factors had a significant impact on DNA damage. Albumin for instance significantly decreased strand breaks in young women < 30 years, indicating that albumin might have an antigenotoxic potential especially in young women. There was a significant positive correlation between uric acid and strand breaks mainly in men. Thus, uric acid induced DNA damage and further creatinine correlated positively with DNA damage.

Regarding gender, it was observed that women show significantly lower amounts of single- and double- strand breaks compared to men. Higher DNA damage in men might be explained by higher uric acid levels which showed

prooxidative effects and lower levels of cobalamin which on the contrary showed protective effect towards DNA damage.

Age on the other hand showed no significant impact on DNA damage, when measured with this assay.

There were also dietary factors which influenced DNA stability significantly. It is necessary to mention the essential water-soluble Vitamin B₁₂ (cobalamin). Individuals with high levels of vitamin B₁₂ showed significantly lower DNA damage in comparison to people with low levels of vitamin B₁₂ levels. Also folic acid showed an impact on DNA damage although results are controversial. Folic acid partly induced DNA damage but also reduced DNA damage depending on the study population analysed.

In conclusion there were various factors which influenced biomarkers of oxidative DNA damage detected by the comet assay. Such influencing factors were gender; endogenous compounds mainly albumin, uric acid and creatinine but also dietary substances like vitamin B₁₂ and folic acid. However, we were not able to validate the antioxidant capabilities of bilirubin towards at DNA damage or protection against it in subjects with GS. Further investigations are necessary to get more insight into the potent antioxidant activities of bilirubin at DNA level.

6. Summary

This present work was carried out at the Department of Nutritional Sciences at the University of Vienna and was funded by the FWF-Austria Science Fund. The major objective of this work was to evaluate the antigenotoxic potential of unconjugated bilirubin in a large human case-control study.

Therefore persons with mild elevated levels of unconjugated bilirubin (Gilbert's syndrome, $n = 45$) as well as individuals with normal bilirubin levels (control, $n = 39$) were recruited from the general population. According to their level of unconjugated bilirubin, which was measured by HPLC, subjects were allocated to the Gilbert or control group. Inclusion and exclusion criteria were checked by a blood sample and questionnaires beforehand. Subjects were recruited by information sheets and advertisements in public buildings and internet platforms. The study was approved by the Human Ethics Research Committee of the general hospital, Vienna (Ref. No.: 274/2010).

To measure DNA damage biomarkers a commonly applied method the "Comet assay" was conducted. In order to attain the level of oxidative DNA damage additionally specific repair enzymes were included (ENDO III and FPG).

Bilirubin has already been reported to have antioxidant properties. As a consequence it was hypothesised that individuals with elevated bilirubin concentrations show less DNA damage than controls. However, the hypothesis could not be confirmed as there were no differences concerning DNA damage in Gilbert and control.

In particular it was observed that other endogenous factors showed significant impact on DNA damage, but bilirubin did not. Serum albumin showed an inverse relation with DNA damage, whereas uric acid and creatinine induced DNA damage and subsequently showed prooxidant effects. Additionally a strong negative correlation was found between vitamin B₁₂ and DNA damage. In

respect of gender, women showed significantly lower amounts of DNA strand breaks compared to men. Age showed no influence on DNA damage.

So far, there is only very little information available on the impact of elevated bilirubin levels on DNA damage. In order to investigate the beneficial function of bilirubin at DNA level further research is required, focusing more on risk groups of oxidative stress.

7. Zusammenfassung

Die vorliegende Arbeit wurde am Institut für Ernährungswissenschaften an der Universität Wien durchgeführt und vom Österreichischen Wissenschaftsfond unterstützt. Hauptziel dieser Arbeit war es das antigenotoxische Potential von unkonjugiertem Bilirubin in einer großen Fall-Kontroll Studie zu erfassen.

Dafür wurden Personen mit leicht erhöhtem unkonjugiertem Bilirubin (Gilbert's syndrom, n= 45) sowie Personen mit normalem Bilirubinspiegel (Kontrolle, n= 39) von der allgemeinen Bevölkerung rekrutiert. Die Probanden wurden je nach unkonjugiertem Bilirubinspiegel welcher mittels HPLC erfasst wurde zu der Gruppe Gilbert's syndrom oder Kontrolle zugeordnet. Ein- und Ausschlusskriterien wurden mit Hilfe einer Blutprobe sowie durch Fragebögen überprüft. Die Rekrutierung der Testpersonen erfolgte mit Hilfe von Merkblättern und Anzeigen in öffentlichen Gebäuden sowie Internetplattformen. Die Studie wurde von der Ethikkommission des Allgemeinen Krankenhauses der Stadt Wien genehmigt (Nr.: 274/2010).

Zur Messung von Biomarkern von DNA-Schäden wurde eine häufig verwendete Methode nämlich der „Comet assay“ eingesetzt. Zusätzlich wurden Reparaturenzyme miteinbezogen, um das Niveau von oxidativen DNA-Schäden zu ermitteln.

Bilirubin konnte in verschiedenen Studien zeigen, dass es antioxidative Eigenschaften besitzt. Demzufolge wurde die Hypothese aufgestellt, dass Personen mit erhöhtem Bilirubinspiegel geringere DNA-Schäden aufweisen als die Kontrollgruppe. Diese Hypothese konnte jedoch nicht bestätigt werden, da es keine Unterschiede in Bezug auf DNA-Schäden zwischen Gilbert und Kontrolle gab.

Im Gegensatz zu Bilirubin wurde jedoch gezeigt, dass andere endogene Faktoren erheblichen Einfluss auf DNA-Schäden aufwiesen. Einerseits zeigte Serumalbumin eine inverse Beziehung mit DNA-Schäden vor allem bei jungen Frauen. Andererseits induzierten Harnsäure und Kreatinin DNA-Schäden und wirkten somit prooxidativ.

Eine starke negative Korrelation wurde zwischen DNA-Schäden und Vitamin B₁₂ beobachtet. In Bezug auf das Geschlecht, zeigten Frauen signifikant geringere DNA-Schäden als Männer. Das Alter hingegen hatte keinen Einfluss auf die DNA-Stabilität.

Bis zum jetzigen Zeitpunkt gibt es sehr wenig Information über den Einfluss von Bilirubin auf die DNA-Stabilität in Personen mit GS. Um die Schutzfunktion von Bilirubin auf die DNA genauer zu untersuchen sind weitere Forschungsarbeiten erforderlich, dabei sollte man sich auf spezielle Risikogruppen fokussieren.

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9. Appendix

a. Comparison of DNA damage in Gilbert's syndrome and Control

a.1. Differences in GS and Control

Table: % DNA in tail in GS and control (all test subjects)

	Gilbert's syndrome	Control	pValue
Number	45	37	
% DNA in tail Lysis	5.3 ± 1.35	5.0 ± 1.18	0.252
% DNA in tail FPG	5.3 ± 3.13	4.6 ± 2.32	0.231
% DNA in tail ENDO III	2.1 ± 1.17	2.5 ± 1.38	0.245

a.2. Differences in GS and Control stratified for gender

Table: % DNA in tail in GS and control (men and women)

	Gilbert's syndrome	Control	pValue
Men			
Number	35	22	
% DNA in tail Lysis	5.5 ± 1.27	5.1 ± 1.23	0.245
% DNA in tail FPG	5.4 ± 3.28	5.0 ± 2.46	0.588
% DNA in tail ENDO III	2.2 ± 1.05	2.7 ± 1.38	0.157
Women			
Number	10	15	
% DNA in tail Lysis	4.5 ± 1.35	4.7 ± 1.12	0.626
% DNA in tail FPG	4.9 ± 2.65	3.9 ± 2.03	0.326
% DNA in tail ENDO III	2.0 ± 1.54	1.38 ± 1.38	0.749

a.3. Differences in GS and Control stratified for age

Table: % DNA in tail in GS and control (< 30 years and ≥ 30 years)

	Gilbert's syndrome	Control	pValue
< 30			
Number	26	23	
% DNA in tail Lysis	5.2 ± 1.35	4.8 ± 1.14	0.217
% DNA in tail FPG	5.9 ± 3.74	5.1 ± 2.33	0.353
% DNA in tail ENDO III	2.2 ± 1.07	2.5 ± 1.07	0.360
≥ 30			
Number	19	14	
% DNA in tail Lysis	5.4 ± 1.37	5.3 ± 1.21	0.831
% DNA in tail FPG	4.5 ± 1.84	3.7 ± 2.1	0.267
% DNA in tail ENDO III	2.1 ± 1.31	3.4 ± 1.49	0.540

a.4. Differences in GS and Control stratified for age and gender

Table: % DNA in tail in GS and control (stratified for age and gender)

	Gilbert's syndrome	Control	pValue
Men < 30			
Number	22	14	
% DNA in tail Lysis	5.4 ± 1.31	5.0 ± 1.13	0.300
% DNA in tail FPG	6.1 ± 3.82	5.7 ± 2.26	0.635
% DNA in tail ENDO III	2.4 ± 1.02	2.8 ± 1.27	0.297
Men ≥ 30			
Number	13	8	
% DNA in tail Lysis	5.7 ± 1.23	5.4 ± 1.42	0.598
% DNA in tail FPG	4.25 ± 1.59	3.8 ± 2.49	0.648
% DNA in tail ENDO III	2.0 ± 1.09	2.5 ± 1.62	0.381
Women < 30			
Number	4	9	
% DNA in tail Lysis	4.1 ± 1.04	4.4 ± 1.14	0.606
% DNA in tail FPG	4.5 ± 3.38	4.1 ± 2.25	0.815
% DNA in tail ENDO III	1.5 ± 1.14	2.17 ± 1.46	0.432
Women ≥ 30			
Number	6	6	
% DNA in tail Lysis	4.8 ± 1.55	5.2 ± 0.98	0.575
% DNA in tail FPG	5.1 ± 2.36	3.6 ± 1.78	0.241
% DNA in tail ENDO III	2.3 ± 1.78	2.2 ± 1.37	0.906

b. Comparison of DNA damage in bilirubin tertiles

b.1. Differences in bilirubin tertiles

Table: % DNA in tail in bilirubin tertiles (all test subjects)

	Tertile 1: 0.24-0.65 mg/dl	Tertile 2: 0.66 -1.21 mg/dl	Tertile 3: 1.22 -4.3 mg/dl	pValue
Number	31	34	33	
% DNA in tail Lysis	4.9 ± 1.21	5.3 ± 1.14	5.1 ± 1.33	0.328
% DNA in tail FPG	4.7 ± 2.42	5.1 ± 3.08	4.7 ± 2.81	0.833
% DNA in tail ENDO III	2.3 ± 1.29	1.9 ± 1.32	2.2 ± 1.12	0.410

b.2. Differences in bilirubin tertiles stratified for gender

Table: % DNA in tail in bilirubin tertiles (men and women)

	Tertile 1: 0.24-0.65 mg/dl	Tertile 2: 0.66 -1.21 mg/dl	Tertile 3: 1.22 -4.3 mg/dl	pValue
Men				
Number	16	26	27	
% DNA in tail Lysis	5.3 ± 1.31	5.5 ± 1.12	5.3 ± 1.26	0.834
% DNA in tail FPG	5.2 ± 2.76	5.2 ± 3.13	4.9 ± 2.95	0.903
% DNA in tail ENDO III	2.6 ± 1.33	2.1 ± 1.33	2.2 ± 0.98	0.397
Women				
Number	15	8	6	
% DNA in tail Lysis	4.5 ± 0.95	4.9 ± 1.19	4.5 ± 1.58	0.622
% DNA in tail FPG	4.2 ± 1.99	4.8 ± 3.07	4.3 ± 2.25	0.832
% DNA in tail ENDO III	2.1 ± 1.23	1.5 ± 1.26	2.6 ± 1.68	0.337

b.3. Differences in bilirubin tertiles stratified for age

Table: % DNA in tail in bilirubin tertiles (< 30 years and ≥ 30 years)

	Tertile 1: 0.24-0.65 mg/dl	Tertile 2: 0.66 -1.21 mg/dl	Tertile 3: 1.22 -4.3 mg/dl	pValue
< 30				
Number	23	21	17	
% DNA in tail Lysis	4.6 ± 1.04	5.6 ± 1.10	4.8 ± 1.29	0.016
% DNA in tail FPG	5.0 ± 2.34	5.6 ± 3.40	5.0 ± 3.54	0.781
% DNA in tail ENDO III	2.5 ± 1.33	1.9 ± 1.24	2.2 ± 0.99	0.239
≥ 30				
Number	8	13	16	
% DNA in tail Lysis	5.6 ± 1.45	4.9 ± 1.13	5.5 ± 1.31	0.389
% DNA in tail FPG	3.9 ± 2.66	4.3 ± 2.35	4.5 ± 1.83	0.854
% DNA in tail ENDO III	1.9 ± 1.09	2.1 ± 1.50	2.3 ± 1.26	0.767

b.4. Differences in bilirubin tertiles stratified for age and gender

Table: % DNA in tail in bilirubin tertiles (stratified for age and gender)

	Tertile 1: 0.24-0.65 mg/dl	Tertile 2: 0.66 -1.21 mg/dl	Tertile 3: 1.22 -4.3 mg/dl	pValue
Men < 30				
Number	12	17	15	
% DNA in tail Lysis	5.2 ± 1.6	5.6 ± 1.18	4.99 ± 1.22	0.310
% DNA in tail FPG	5.6 ± 2.45	5.6 ± 3.41	5.3 ± 3.69	0.943
% DNA in tail ENDO III	2.8 ± 1.38	2.0 ± 1.26	2.2 ± 0.94	0.266
Men ≥ 30				
Number	4	9	12	
% DNA in tail Lysis	5.6 ± 2.09	5.2 ± 1.02	5.7 ± 1.26	0.726
% DNA in tail FPG	3.9 ± 3.59	4.3 ± 2.47	4.3 ± 1.63	0.946
% DNA in tail ENDO III	2.0 ± 1.18	2.2 ± 1.51	2.1 ± 1.05	0.970
Women < 30				
Number	11	4	2	
% DNA in tail Lysis	4.0 ± 0.63	5.6 ± 0.79	3.3 ± 0.83	0.002
% DNA in tail FPG	4.3 ± 2.11	5.4 ± 3.88	3.1 ± 0.99	0.560
% DNA in tail ENDO III	2.2 ± 1.28	1.2 ± 1.00	1.7 ± 1.69	0.421
Women ≥ 30				
Number	4	4	4	
% DNA in tail Lysis	5.6 ± 0.71	4.3 ± 1.29	5.1 ± 1.58	0.380
% DNA in tail FPG	3.93 ± 1.89	4.2 ± 2.43	4.8 ± 2.60	0.855
% DNA in tail ENDO III	1.7 ± 1.17	1.8 ± 1.74	3.0 ± 1.73	0.486

c. Correlations

c.1. Correlation between lysis, FPG, ENDO III, BMI, LDL, HDL and TG

Table: Correlation between lysis, FPG, ENDO III, BMI, LDL, HDL and TG (all test subjects)

All test subjects		Lysis	FPG	ENDO III	BMI	LDL	HDL	TG
Lysis	r	1						
	p							
FPG	r	0.387**	1					
	p	0.000						
ENDO III	r	0.183	0.289**	1				
	p	0.077	0.005					
BMI	r	0.175	-0.042	-0.003	1			
	p	0.085	0.678	0.975				
LDL	r	-0.079	-0.160	0.002	0.363**	1		
	p	0.439	0.116	0.984	0.000			
HDL	r	-0.164	0.020	0.076	-0.240*	-0.063	1	
	p	0.106	0.848	0.468	0.016	0.535		
TG	r	0.025	-0.035	-0.013	0.364**	0.248*	-0.133	1
	p	0.808	0.734	0.902	0.000	0.013	0.186	

Table: Correlation between lysis, FPG, ENDO III, BMI, LDL, HDL and TG (men)

Men		Lysis	FPG	ENDO III	BMI	LDL	HDL	TG
Lysis	r	1						
	p							
FPG	r	0.362**	1					
	p	0.002						
ENDO III	r	0.269*	0.272*	1				
	p	0.028	0.026					
BMI	r	0.277*	-0.048	0.073	1			
	p	0.021	0.695	0.559				
LDL	r	-0.074	-0.210	-0.060	0.348**	1		
	p	0.547	0.083	0.627	0.003			
HDL	r	-0.137	-0.016	0.009	-0.177	-0.030	1	
	p	0.261	0.893	0.943	0.143	0.808		
TG	r	0.093	-0.042	0.000	0.372**	0.290*	-0.277*	1
	p	0.445	0.732	0.997	0.002	0.015	0.020	

Table: Correlation between lysis, FPG, ENDO III, BMI, LDL, HDL and TG (women)

Women		Lysis	FPG	ENDO III	BMI	LDL	HDL	TG
Lysis	r	1						
	p							
FPG	r	0.376*	1					
	p	0.044						
ENDO III	r	-0.069	0.331	1				
	p	0.732	0.091					
BMI	r	-0.080	-0.059	-0.187	1			
	p	0.680	0.760	0.351				
LDL	r	-0.245	-0.029	0.148	0.401*	1		
	p	0.200	0.880	0.461	0.028			
HDL	r	0.081	0.268	0.301	-0.291	0.015	1	
	p	0.677	0.161	0.128	0.119	0.938		
TG	r	-0.069	0.015	-0.034	0.375*	0.169	-0.021	1
	p	0.721	0.939	0.867	0.041	0.372	0.911	

Table: Correlation between lysis, FPG, ENDO III, BMI, LDL, HDL and TG (< 30 years)

<30 years		Lysis	FPG	ENDO III	BMI	LDL	HDL	TG
Lysis	r	1						
	p							
FPG	r	0.382**	1					
	p	0.002						
ENDO III	r	0.165	0.374**	1				
	p	0.212	0.003					
BMI	r	0.141	0.035	0.179	1			
	p	0.278	0.788	0.175				
LDL	r	-0.119	-0.043	-0.012	0.217	1		
	p	0.361	0.740	0.931	0.092			
HDL	r	-0.229	-0.003	0.079	-0.138	-0.182	1	
	p	0.076	0.981	0.551	0.289	0.160		
TG	r	-0.032	-0.100	-0.011	0.368**	0.083	-0.040	1
	p	0.807	0.441	0.933	0.004	0.524	0.759	

Table: Correlation between lysis, FPG, ENDO III, BMI, LDL, HDL and TG (≥ 30 years)

≥ 30 years		Lysis	FPG	ENDO III	BMI	LDL	HDL	TG
Lysis	r	1						
	p							
FPG	r	0.485**	1					
	p	0.002						
ENDO III	r	0.220	0.116	1				
	p	0.204	0.505					
BMI	r	0.151	-0.042	-0.271	1			
	p	0.371	0.806	0.115				
LDL	r	-0.102	-0.303	0.028	-0.429**	1		
	p	0.547	0.068	0.874	0.006			
HDL	r	-0.116	0.133	0.074	-0.471**	0.010	1	
	p	0.494	0.433	0.673	0.002	0.951		
TG	r	0.093	0.120	-0.015	0.361*	0.442**	-0.267	1
	p	0.584	0.481	0.931	0.024	0.005	0.101	

Table: Correlation between lysis, FPG, ENDO III, BMI, LDL, HDL and TG (men < 30 years)

Men < 30 years		Lysis	FPG	ENDO III	BMI	LDL	HDL	TG
Lysis	r	1						
	p							
FPG	r	0.382*	1					
	p	0.011						
ENDO III	r	0.329*	0.378*	1				
	p	0.033	0.014					
BMI	r	0.195	-0.041	0.195	1			
	p	0.205	0.792	0.215				
LDL	r	-0.093	-0.097	-0.176	0.252	1		
	p	0.550	0.530	0.264	0.099			
HDL	r	-0.033	0.103	-0.017	-0.116	-0.240	1	
	p	0.830	0.506	0.916	0.453	0.117		
TG	r	0.029	-0.152	0.036	0.366*	0.195	-0.293	1
	p	0.853	0.324	0.822	0.015	0.205	0.053	

Table: Correlation between lysis, FPG, ENDO III, BMI, LDL, HDL and TG (men ≥ 30 years)

Men ≥ 30 years		Lysis	FPG	ENDO III	BMI	LDL	HDL	TG
Lysis	r	1						
	p							
FPG	r	0.431*	1					
	p	0.031						
ENDO III	r	0.191	0.000	1				
	p	0.361	1.000					
BMI	r	0.416*	0.282	-0.065	1			
	p	0.039	0.172	0.758				
LDL	r	-0.113	-0.318	0.178	0.263	1		
	p	0.591	0.122	0.395	0.195			
HDL	r	-0.338	-0.305	0.069	-0.417*	0.291	1	
	p	0.099	0.139	0.743	0.034	0.149		
TG	r	0.149	0.249	-0.025	0.326	0.332	-0.291	1
	p	0.476	0.230	0.907	0.104	0.097	0.149	

Table: Correlation between lysis, FPG, ENDO III, BMI, LDL, HDL and TG (women < 30 years)

Women < 30 years		Lysis	FPG	ENDO III	BMI	LDL	HDL	TG
Lysis	r	1						
	p							
FPG	r	0.234	1					
	p	0.365						
ENDO III	r	-0.499*	0.323	1				
	p	0.041	0.206					
BMI	r	0.007	0.305	0.134	1			
	p	0.980	0.234	0.608				
LDL	r	-0.336	0.176	0.521*	0.097	1		
	p	0.187	0.500	0.032	0.711			
HDL	r	-0.319	-0.061	0.452	-0.214	-0.043	1	
	p	0.212	0.817	0.069	0.409	0.869		
TG	r	0.093	0.133	-0.018	0.410	-0.171	0.147	1
	p	0.722	0.610	0.944	0.102	0.511	0.574	

Table: Correlation between lysis, FPG, ENDO III, BMI, LDL, HDL and TG (women ≥ 30 years)

Women ≥ 30 years		Lysis	FPG	ENDO III	BMI	LDL	HDL	TG
Lysis	r	1						
	p							
FPG	r	0.645*	1					
	p	0.024						
ENDO III	r	0.318	0.366	1				
	p	0.370	0.298					
BMI	r	-0.241	-0.452	-0.538	1			
	p	0.451	0.140	0.109				
LDL	r	-0.245	-0.286	-0.275	0.601*	1		
	p	0.442	0.367	0.442	0.030			
HDL	r	0.352	0.751**	0.046	-0.406	0.034	1	
	p	0.262	0.005	0.900	0.168	0.913		
TG	r	-0.174	-0.289	0.074	0.482	0.776**	-0.190	1
	p	0.589	0.362	0.839	0.095	0.002	0.534	

c.2. Correlations between lysis, FPG, ENDO III, creatinine, uric acid, albumin

Table: Correlation between lysis, FPG, ENDO III, creatinine, uric acid and albumin (all test subjects)

All test subjects		Lysis	FPG	ENDO III	Creatinine	Uric acid	Albumin
Lysis	r	1					
	p						
FPG	r	0.378**	1				
	p	0.000					
ENDO III	r	0.183	0.289**	1			
	p	0.077	0.005				
Creatinine	r	0.298**	0.164	0.086	1		
	p	0.003	0.107	0.413			
Uric acid	r	0.426**	0.213*	0.092	0.537**	1	
	p	0.000	0.035	0.377	0.000		
Albumin	r	-0.137	0.037	0.110	0.302**	0.235*	1
	p	0.178	0.716	0.290	0.002	0.019	

Table: Correlation between lysis, FPG, ENDO III, creatinine, uric acid and albumin (men)

Men		Lysis	FPG	ENDO III	Creatinine	Uric acid	Albumin
Lysis	r	1					
	p						
FPG	r	0.362**	1				
	p	0.002					
ENDO III	r	0.269*	0.272*	1			
	p	0.028	0.026				
Creatinine	r	0.106	0.076	0.099	1		
	p	0.385	0.532	0.428			
Uric acid	r	0.411**	0.1867	0.135	0.221	1	
	p	0.000	0.125	0.275	0.066		
Albumin	r	-0.244*	0.003	0.008	0.176	-0.015	1
	p	0.044	0.980	0.950	0.144	0.900	

Table: Correlation between lysis, FPG, ENDO III, creatinine, uric acid and albumin (women)

Women		Lysis	FPG	ENDO III	Creatinine	Uric acid	Albumin
Lysis	r	1					
	p						
FPG	r	0.376*	1				
	p	0.044					
ENDO III	r	-0.069	0.331	1			
	p	0.732	0.091				
Creatinine	r	0.308	0.336	-0.102	1		
	p	0.104	0.075	0.613			
Uric acid	r	0.108	0.184	-0.136	0.042	1	
	p	0.578	0.338	0.498	0.824		
Albumin	r	-0.387*	-0.038	0.237	-0.332	0.005	1
	p	0.038	0.846	0.234	0.073	0.980	

Table: Correlation between lysis, FPG, ENDO III, creatinine, uric acid and albumin (< 30 years)

< 30 years		Lysis	FPG	ENDO III	Creatinine	Uric acid	Albumin
Lysis	r	1					
	p						
FPG	r	0.382**	1				
	p	0.002					
ENDO III	r	0.165	0.374**	1			
	p	0.212	0.003				
Creatinine	r	0.362**	0.218	0.108	1		
	p	0.004	0.092	0.414			
Uric acid	r	0.533**	0.245	0.215	0.632**	1	
	p	0.000	0.057	0.102	0.000		
Albumin	r	-0.072	0.028	0.141	0.385**	0.302*	1
	p	0.579	0.828	0.287	0.002	0.018	

Table: Correlation between lysis, FPG, ENDO III, creatinine, uric acid and albumin (≥ 30 years)

≥ 30 years		Lysis	FPG	ENDO III	Creatinine	Uric acid	Albumin
Lysis	r	1					
	p						
FPG	r	0.485**	1				
	p	0.002					
ENDO III	r	0.220	0.116	1			
	p	0.204	0.505				
Creatinine	r	0.211	0.035	0.049	1		
	p	0.210	0.835	0.782			
Uric acid	r	0.273	0.119	-0.139	0.371*	1	
	p	0.102	0.482	0.427	0.020		
Albumin	r	-0.158	-0.116	0.061	0.165	0.096	1
	p	0.351	0.494	0.727	0.315	0.561	

Table: Correlation between lysis, FPG, ENDO III, creatinine, uric acid and albumin (women < 30 years)

Women < 30 years		Lysis	FPG	ENDO III	Creatinine	Uric acid	Albumin
Lysis	r	1					
	p						
FPG	r	0.234	1				
	p	0.365					
ENDO III	r	-0.499*	0.323	1			
	p	0.041	0.206				
Creatinine	r	0.231	0.313	-0.277	1		
	p	0.372	0.221	0.282			
Uric acid	r	0.293	0.214	-0.013	0.160	1	
	p	0.254	0.410	0.960	0.539		
Albumin	r	-0.565*	-0.021	0.382	-0.228	-0.384	1
	p	0.018	0.935	0.130	0.378	0.128	

Table: Correlation between lysis, FPG, ENDO III, creatinine, uric acid and albumin (women ≥ 30 years)

Women ≥ 30 years		Lysis	FPG	ENDO III	Creatinine	Uric acid	Albumin
Lysis	r	1					
	p						
FPG	R	0.645*	1				
	p	0.024					
ENDO III	R	0.318	0.366	1			
	p	0.370	0.298				
Creatinine	R	0.424	0.377	0.106	1		
	p	0.169	0.226	0.771			
Uric acid	R	-0.131	0.192	-0.348	-0.034	1	
	p	0.685	0.551	0.324	0.913		
Albumin	R	-0.153	-0.078	-0.024	-0.514	0.453	1
	p	0.636	0.810	0.947	0.073	0.120	

Table: Correlation between lysis, FPG, ENDO III, creatinine, uric acid and albumin (men < 30 years)

Men < 30 years		Lysis	FPG	ENDO III	Creatinine	Uric acid	Albumin
Lysis	r	1					
	p						
FPG	r	0.382*	1				
	p	0.011					
ENDO III	R	0.329*	0.378*	1			
	p	0.033	0.014				
Creatinine	R	0.149	0.121	0.092	1		
	p	0.334	0.434	0.563			
Uric acid	R	0.444**	0.184	0.211	0.352*	1	
	p	0.003	0.233	0.180	0.019		
Albumin	R	-0.245	-0.102	-0.152	0.165	0.047	1
	p	0.110	0.509	0.337	0.283	0.763	

Table: Correlation between lysis,FPG, ENDO III, creatinine, uric acid and albumin (men ≥ 30 years)

Men ≥ 30 years		Lysis	FPG	ENDO III	Creatinine	Uric acid	Albumin
Lysis	R	1					
	p						
FPG	R	0.431*	1				
	p	0.031					
ENDO III	R	0.191	0.000	1			
	p	0.361	1.000				
Creatinine	R	0.028	-0.029	0.120	1		
	p	0.893	0.892	0.567			
Uric acid	R	0.389	0.132	-0.024	-0.039	1	
	p	0.055	0.529	0.911	0.850		
Albumin	R	-0.233	-0.132	0.112	0.233	-0.243	1
	p	0.263	0.530	0.593	0.252	0.231	

c.3. Correlations between lysis, FPG, ENDO III, age, UCB, iron, vitamin C and glutathione

Table: Correlation between lysis, FPG, ENDO III, age, UCB, iron, vitamin C and glutathione (all test subjects)

All test subjects		Lysis	FPG	ENDO III	Age	UCB	Iron	Vitamin C	Glutathione
Lysis	r	1							
	p								
FPG	r	0.387**	1						
	p	0.000							
ENDO III	r	0.183	0.289**	1					
	p	0.077	0.005						
Age	r	0.077	-0.090	-0.070	1				
	p	0.452	0.378	0.506					
UCB	r	0.055	0.094	-0.050	0.125	1			
	p	0.592	0.356	0.634	0.216				
Iron	r	-0.027	-0.034	-0.061	-0.093	0.480**	1		
	p	0.794	0.738	0.562	0.360	0.000			
Vitamin C	r	0.057	0.099	0.111	-0.113	-0.085	0.151	1	
	p	0.578	0.334	0.286	0.263	0.403	0.135		
Glutathione	r	0.225*	0.031	0.105	0.149	-0.051	-0.072	0.102	1
	p	0.026	0.764	0.313	0.140	0.618	0.481	0.314	

Table: Correlation between lysis, FPG, ENDO III, age, UCB, iron, vitamin C and glutathione (men)

Men		Lysis	FPG	ENDO III	Age	UCB	Iron	Vitamin C	Glutathione
Lysis	r	1							
	p								
FPG	r	0.362**	1						
	p	0.002							
ENDO III	r	0.269*	0.272*	1					
	p	0.028	0.026						
Age	r	0.005	-0.131	-0.138	1				
	p	0.967	0.85	0.266					
UCB	r	-0.013	0.083	-0.099	0.153	1			
	p	0.913	0.500	0.427	0.207				
Iron	r	-0.131	-0.126	-0.210	-0.111	0.457**	1		
	p	0.288	0.305	0.090	0.364	0.000			
Vitamin C	r	0.118	0.127	0.030	-0.107	-0.151	0.080	1	
	p	0.335	0.298	0.810	0.380	0.212	0.512		
Glutathione	r	0.309**	0.107	0.065	0.065	-0.019	-0.118	0.086	1
	p	0.010	0.380	0.604	0.591	0.875	0.334	0.478	

Table: Correlation between lysis, FPG, ENDO III, age, UCB, iron, vitamin C and glutathion (women)

Women		Lysis	FPG	ENDO III	Age	UCB	Iron	Vitamin C	Glutathion
Lysis	r	1							
	p								
FPG	r	0.376*	1						
	p	0.044							
ENDO III	r	-0.069	0.331	1					
	p	0.732	0.091						
Age	r	0.326	0.051	0.083	1				
	p	0.084	0.793	0.680					
UCB	r	-0.050	0.002	0.021	0.142	1			
	p	0.795	0.991	0.916	0.453				
Iron	r	-0.061	0.113	0.186	0.006	0.400	1		
	p	0.753	0.559	0.353	0.973	0.029			
Vitamin C	r	0.010	0.068	0.301	-0.146	0.192	0.384*	1	
	p	0.960	0.728	0.127	0.443	0.310	0.036		
Glutathion	r	0.315	-0.081	0.250	0.319	0.093	0.212	0.093	1
	p	0.096	0.676	0.208	0.092	0.631	0.270	0.633	

Table: Correlation between lysis, FPG, ENDO III, age, UCB, iron, vitamin C and glutathion (< 30 years)

< 30 years		Lysis	FPG	ENDO III	Age	UCB	Iron	Vitamin C	Glutathion
Lysis	r	1							
	p								
FPG	r	0.382**	1						
	p	0.002							
ENDO III	r	0.165	0.374**	1					
	p	0.212	0.003						
Age	r	-0.014	-0.067	-0.022	1				
	p	0.914	0.607	0.867					
UCB	r	-0.020	0.148	-0.111	0.124	1			
	p	0.878	0.255	0.403	0.341				
Iron	r	-0.002	-0.060	-0.148	0.108	0.541**	1		
	p	0.986	0.648	0.263	0.407	0.000			
Vitamin C	r	0.185	0.138	-0.031	0.077	0.047	0.195	1	
	p	0.153	0.290	0.818	0.553	0.720	0.133		
Glutathion	r	0.331**	0.199	0.090	-0.273*	0.083	-0.146	0.078	1
	p	0.009	0.124	0.499	0.033	0.526	0.260	0.548	

Table: Correlation between lysis, FPG, ENDO III, age, UCB, iron, vitamin C and glutathion (≥ 30 years)

≥ 30 years		Lysis	FPG	ENDO III	Age	UCB	Iron	Vitamin C	Glutathion
Lysis	r	1							
	p								
FPG	r	0.485**	1						
	p	0.002							
ENDO III	r	0.220	0.116	1					
	p	0.204	0.505						
Age	r	-0.044	0.178	-0.150	1				
	p	0.796	0.293	0.391					
UCB	r	0.124	0.061	0.033	0.083	1			
	p	0.466	0.720	0.852	0.616				
Iron	r	-0.050	-0.014	0.129	-0.078	0.387*	1		
	p	0.773	0.936	0.467	0.640	0.016			
Vitamin C	r	-0.096	-0.026	0.329	-0.172	-0.239	0.054	1	
	p	0.574	0.879	0.054	0.295	0.142	0.747		
Glutathion	r	0.014	-0.085	0.159	-0.337*	-0.329*	0.192	0.235	1
	p	0.934	0.615	0.362	0.039	0.044	0.255	0.155	

Table: Correlation between lysis, FPG, ENDO III, age, UCB, iron, vitamin C and glutathion (women < 30 years)

Women < 30 years		Lysis	FPG	ENDO III	Age	UCB	Iron	Vitamin C	Glutathion
Lysis	r	1							
	p								
FPG	r	0.234	1						
	p	0.365							
ENDO III	r	-0.499*	0.323	1					
	p	0.041	0.206						
Age	r	-0.014	-0.393	0.010	1				
	p	0.958	0.119	0.968					
UCB	r	-0.098	-0.060	-0.179	0.131	1			
	p	0.710	0.820	0.493	0.617				
Iron	r	-0.025	0.110	-0.100	-0.048	0.516*	1		
	p	0.926	0.675	0.704	0.855	0.034			
Vitamin C	r	0.018	-0.037	0.066	0.106	0.327	0.585*	1	
	p	0.946	0.888	0.800	0.687	0.200	0.014		
Glutathion	r	0.087	-0.143	0.145	-0.081	0.164	-0.063	0.038	1
	p	0.741	0.584	0.578	0.756	0.529	0.811	0.884	

Table: Correlation between lysis, FPG, ENDO III, age, UCB, iron, vitamin C and glutathion (women ≥ 30 years)

Women ≥ 30 years		Lysis	FPG	ENDO III	Age	UCB	Iron	Vitamin C	Glutathion
Lysis	r	1							
	p								
FPG	r	0.645*	1						
	p	0.024							
ENDO III	r	0.318	0.366	1					
	p	0.370	0.298						
Age	r	0.195	0.394	-0.055	1				
	p	0.544	0.205	0.881					
UCB	r	-0.136	0.111	0.206	-0.037	1			
	p	0.674	0.732	0.568	0.906				
Iron	r	-0.156	0.126	0.567	-0.062	0.260	1		
	p	0.629	0.696	0.087	0.841	0.390			
Vitamin C	r	0.135	0.212	0.654*	-0.039	0.114	0.188	1	
	p	0.677	0.508	0.040	0.900	0.711	0.538		
Glutathion	r	0.262	-0.016	0.297	-0.433	-0.176	0.459	0.432	1
	p	0.411	0.960	0.405	0.160	0.583	0.133	0.161	

Table: Correlation between lysis, FPG, ENDO III, age, UCB, iron, vitamin C and glutathion (men < 30 years)

Men < 30 years		Lysis	FPG	ENDO	Age	UCB	Iron	Vitamin C	Glutathion
Lysis	r	1							
	p								
FPG	r	0.382*	1						
	p	0.011							
ENDO III	r	0.329*	0.378*	1					
	p	0.033	0.014						
Age	r	-0.052	-0.004	-0.054	1				
	p	0.737	0.982	0.736					
UCB	r	-0.147	0.142	-0.150	0.104	1			
	p	0.341	0.356	0.344	0.503				
Iron	r	-0.156	-0.170	-0.236	0.128	0.497**	1		
	p	0.313	0.269	0.133	0.406	0.001			
Vitamin C	r	0.354*	0.236	-0.040	0.085	0.029	0.138	1	
	p	0.018	0.123	0.801	0.583	0.852	0.372		
Glutathion	r	0.488*	0.306*	0.103	-0.313*	0.116	-0.128	0.069	1
	p	0.001	0.043	0.517	0.038	0.453	0.409	0.658	

Table: Correlation between lysis, FPG, ENDO III, age, UCB, iron, vitamin C and glutathion (men ≥ 30 years)

Men ≥ 30 years		Lysis	FPG	ENDO III	Age	UCB	Iron	Vitamin C	Glutathion
Lysis	r	1							
	p								
FPG	r	0.431*	1						
	p	0.031							
ENDO III	r	0.191	0.000	1					
	p	0.361	1.000						
Age	r	-0.110	0.100	-0.199	1				
	p	0.599	0.634	0.339					
UCB	r	0.147	0.058	-0.007	0.141	1			
	p	0.484	0.785	0.974	0.491				
Iron	r	-0.044	-0.109	-0.191	-0.068	0.442*	1		
	p	0.837	0.613	0.372	0.748	0.027			
Vitamin C	r	-0.230	-0.157	0.139	-0.240	-0.393*	-0.076	1	
	p	0.270	0.455	0.508	0.237	0.047	0.717		
Glutathion	r	-0.007	-0.144	0.071	-0.371	-0.311	0.110	0.155	1
	p	0.973	0.493	0.736	0.062	0.122	0.601	0.451	

10. Curriculum vitae

Personal Information

Name: Nadja Antl
 Email: nadja.antl@hotmail.com
 Date of Birth: April 24th, 1986
 Citizenship: Austria

Education

Since 10/2005	Study of Nutritional Sciences Faculty of Life Sciences, University of Vienna Title of Diploma thesis: <i>"Effects of moderate hyperbilirubinemia on DNA damage"</i> Supervisor: Ao. Univ.-Prof. Dr. Karl-Heinz Wagner
2002-2005	Diploma of the International Baccalauréate Organization, International School of Berne
1997-2002	High School, European School Brussels
1995-1997	Primary School, Munich
1993-1995	Primary School, Vienna
1992-1993	Primary School, Innsbruck

Research activities abroad

02/2011-06/2011	Research visit at the working group of Assistant Professor Ali Moazzami Swedish University of Agricultural Sciences Department of Food Science Division of Plant products, Uppsala (Sweden)
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Work Experience

07/2011	Internship at Children's University, Vienna
12/2010	Internship at institution for food safety (Municipal Department 38) Division of microbiology, Vienna
09/2009	Internship at a food production company Staud's, Vienna
07/2009	Internship at an certified organic farm 'Roots and Greens Farm', British Columbia, Canada
07/2008-09/2008	Internship at a food production company Agrana, Section Fruit products, Division quality control, Lower Austria
04/2008	Internship at the University of Vienna, Department of Nutritional Sciences

Professional Qualifications

- PC: MS Office, SPSS, SIMCA
- Languages: German, English, French, Spanish, Swedish

Further Interests

Winter sports, swimming, traveling, reading

Erklärung zur gemeinsamen Bearbeitung eines Themas

Gemäß dem Universitätsgesetz §81(Abs. 3 UG) wurden die Diplomarbeiten mit dem Titel

„Evaluation of DNA Damage in Gilbert’s Syndrome Subjects in H₂O₂-treated and untreated PBMCs“

und dem zweiten Titel

“Effects of moderate hyperbilirubinemia on DNA damage“

von Barbara Rittmannsberger und Nadja Antl im Rahmen einer Fall-Kontroll-Studie am Institut für Ernährungswissenschaften unter der Betreuung von Herrn Ao. Univ.-Prof. Dr. Karl-Heinz Wagner und Frau Mag. Marlies Wallner an der Universität Wien gemeinsam verfasst.

Im Inhaltsverzeichnis dieser Arbeit sind die Teile der jeweiligen Verfasserin genau zugeordnet.

Unterschrift

Barbara Rittmannsberger (19.08.1984)

Unterschrift

Nadja Antl (24.04.1986)

Wien, Oktober 2011