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"Evaluation of DNA Damage in Gilbert's Syndrome Subjects in H_2O_2-treated and untreated PBMCs"

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Barbara Rittmannsberger

Mitverfasserin: Nadja Antl

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Table of contents

List of figures

List of tables

1. Introduction

This present thesis is part of the research project "Antioxidative and Antigenotoxic Potential of Bile Pigments", promoted by FWF, at the Department of Nutritional Sciences, University of Vienna.

Gilbert's Syndrome (GS) is a condition with increased serum bilirubin levels (around >1 mg/dl) and is found in up to 17 % of the population. It is considered as harmless in adults. GS people have only a 30 % remaining activity of the enzyme hepatic uridine diphosphate glucuronosyltransferase (UGT1A1) and therefore, the circulating unconjugated bilirubin concentration is increasing.

Recent studies already showed that bilirubin, the main bile pigment in organism, has an antioxidative potential in vitro and the ability to decrease oxidation of plasma. In a human study results showed a better protection of serum oxidation in GS compared to normal controls. Therefore, it is speculated that bilirubin works as antioxidant against oxidative stress and furthermore against DNA damage.

The aim of this study was to observe whether people with Gilbert's Syndrome have lower DNA damage than controls. The levels of oxidative DNA damage were measured by Comet assay in PBMCs.

By now there is no literature available concerning GS and Comet assay. In this thesis first results on this background are shown.

Research and lab work was made in collaboration with Nadja Antl and therefore, chapters "Literature survey" and "Material and Methods" were written together and 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 characterized 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 ($^*OH$), superoxide radical (O_2^{*-}) 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 organism play dual roles, deleterious at high concentrations and beneficial roles at low or moderate concentration. Beneficial physiological functions of reactive oxygen species are the defence against pathogens, second messenger in a number of signalling pathways and the induction of mitogenic 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, from peroxisomes during degradation of fatty acids and from leukocytes during the defence mechanism against microbes and from the cytochrome p450 system,

which has mixed functions in the oxidation system. Also, mistakes during cell division can lead to the production of ROS [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 already mentioned free radicals are not deleterious at low or moderate concentrations. In healthy individuals, there is almost a balance maintained between oxidants and antioxidants [VALKO et al., 2006].

Antioxidants can inhibit oxidation of body cells and support the organism to protect DNA by donating e⁻ for free radical detoxification. [MATÉS, 2000; SIES, 1997] Antioxidants are of physiological relevance and can be either enzymatic or non enzymatic. Enzymatic antioxidant systems are for example superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidise (GSH-Px). Non enzymatic antioxidants are vitamin E, vitamin C, carotinoids or serum proteins like uric acid and bilirubin. Some of these antioxidants are synthesised in the body, like glutathione, uric acid and bilirubin, some have to be provided alimentary, like vitamin C, E and β-carotin. [ELMADFA and LEITZMANN, 2004]

An imbalance between oxidants and antioxidants occurs either by excessive production of ROS or deficient capacity of the antioxidants system, leading to an undesirable state, which is called "oxidative stress" (see Figure 1) [COLLINS, 2009].
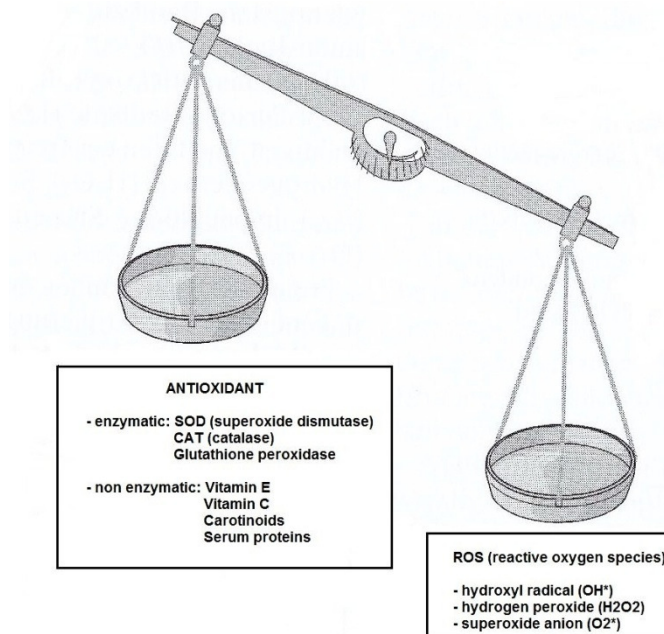
Figure 1: Oxidative Stress (modified after ELMADFA and LEITZMANN, 2004)

During oxidative stress in vivo ROS can attack important biomolecules. Cellular targets for oxidative modification include DNA, lipids and proteins. Which of the biomolecules will be 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]

2.1.3. Oxidative stress and DNA damage

The most important derivate of ROS, which causes damage to DNA is the hydroxyl radical (OH^{*-}). OH^{*-} is the most reactive form of ROS and able to react with all components of the DNA [VALKO 2006]. In the body the OH^{*-} radical can be derived from H_2O_2, which can diffuse across membranes and through the cytosol. At normal state the strong oxidant H_2O_2 is converted into water and molecular oxygen by antioxidant enzymes, like the 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

[*]OH. A direct reaction with DNA is possible and harmful damage can occur. [ROBERTS and SINDHU, 2009]

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 sugars or AP (apurinic/apyrimidinic) sites, DNA-Protein cross links and the modified DNA bases purine and pyrimidine. [EVANS et al., 2004]. DNA strand breaks cannot be specifically related to oxidation since they result from various forms of damage and might also present intermediates in the repair process. In contrary to DNA strand breaks oxidised bases are specific indicators of oxidative damage, reflecting the background damage caused by ROS in the cells [COLLINS, 2009].
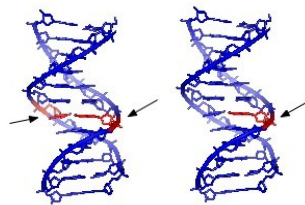
Figure 2: Double and single strand breaks in DNA [http://en.wikipedia.org/wiki/DNA_repair; access date: 20.5.2011]

2.2. Methods for measuring DNA damage

For detecting different endpoints of DNA damage a bunch of methods like the Comet Assay, the micronucleus Assay or 8-OxodG have been developed.

Marker	Method (abbreviation)
8-OxodG	HPLC-ECD, GC/MS, LC-MS/MS
Sister chromatids	Sister chromatid assay (SCEs)
nucleoplasmatic bridges (NPBs) and nuclear buds (NBUDs)	Micronucleus test (CBMN)
Oxidised pyrimidines, 8-OxodG, **Single and Double strand breaks**	Methods with enzymes like **Single cell gel electrophoresis (SCGE) (Comet Assay)**

Table 1: Summary of methods for measuring DNA-damage [WAGNER and JAHREIS,2004; FENECH, 2007; WILCOSKY and RYNARD,1990]

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 added to the heterocyclic structure of guanine at the 8th position (C8-OH-adduct radical) and releases an H-Atom forming the oxidation 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 3) [VALAVANIDIS et al., 2009].
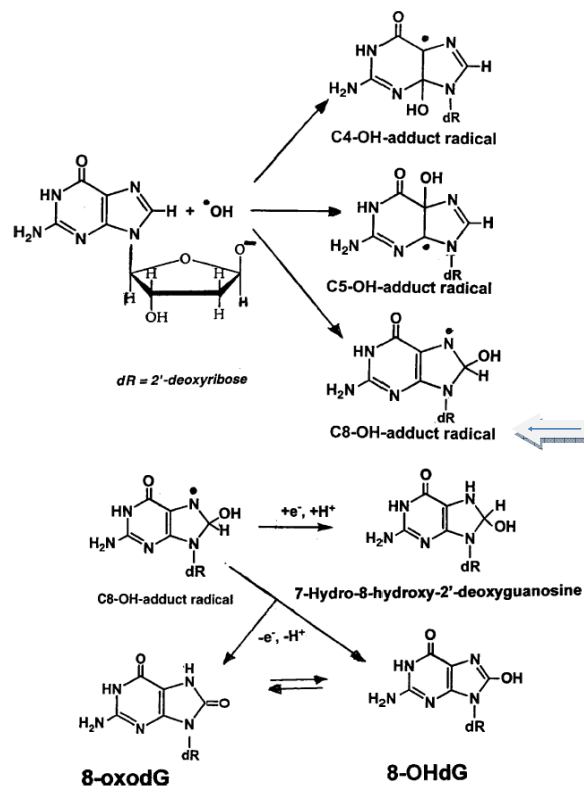
Figure 3: Forming 8-OxodG after reaction of 2´-desoxyguanosine with hydroxyl radicals [VALAVANIDIS et al., 2009]

8-OxodG is one of the most important lesion of DNA caused by free radicals. Therefore, 8-OxodG 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-OxodG 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. (See also Table 1) [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].

> ➤ <u>LC-MS/MS</u> (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 oxidized nucleosides [HENRIKSEN et al., 2009].

2.2.2. Sister chromatid exchange (SCEs)

Sister chromatid exchange (SCEs) is a process that occurs during DNA replication, where two sister chromatids, that are probably genotoxic harmed, break and rejoin with another one. SCEs occur naturally and spontaneous but can also be induced by genotoxic substances and therefore it is a method for measuring cell damage [WILSON and THOMPSON, 2007].

Sister chromatid exchanges can exchange apparently identical segments of DNA without known modification of cell viability or function. The SCEs has been widely used as an in vitro method for measuring the mutagenic potential of chemicals. In the last years interest was growing to use sister chromatid exchange also as marker of DNA lesions in humans that were exposed to certain types of chemical carcinogens. For the visualization of SCEs a different staining at the sister chromatids is important, as for example with the thymidine analog 5-bromodeoxyuridine (BrdU). See also Figure 4. [WILCOSKY and RYNARD, 1990]
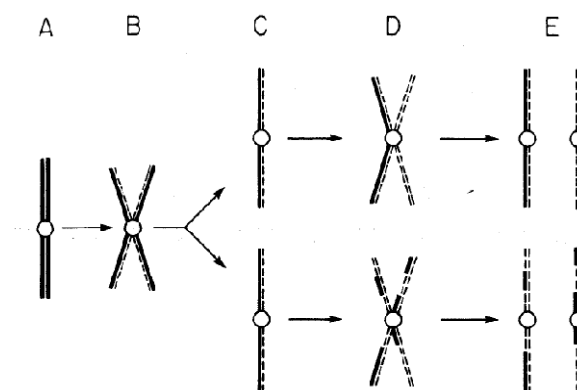
Figure 4: Visualization of SCEs according to Wilcosky and Rynard (1990). [A] Chromosome in G_0-phase [B] First metaphase: After staining with BrdU each sister chromatid consists of one normal parent strand (dark lines) and one BrdU-substituted strand (dashed lines). [C] After mitosis: Two daughter cells. [D] Metaphase in second cell division: daughter cells are composed of one sister chromatid with a parent strand and one BrdU-substituted strand and the other one with both strands out of BrdU-Substitution. [E] Second mitosis. [WILCOSKY and RYNARD, 1990]

2.2.3. Cytokinesis-block micronucleus cytome assay (CBMN)

The cytokinesis-micronucleus (CBMN) assay is a method for measuring micronuclei (MNi), nucleoplasmic buds (NBuds) and nucleoplasmic bridges (NPBs) in cultured human cells.

The micronucleus test in vivo was developed by Schmid and co-workers and is a method for screening chemical exposure and their chromosome-breaking effects [SCHMID, 1975]. The assay has been improved by M. Fenech and colleagues for use in lymphocytes and so today micronucleus test can be used for measuring DNA damage, cytostasis and cytotoxicity [FENECH, 2007].

The micronucleus assay can be used with bone marrow, peripheral blood erythrocytes, peripheral blood lymphocytes and buccal cells and is now one of the best established in vivo cytogenetic assays [FENECH, 2000].

Micronuclei are formations of small membrane bound DNA fragments or whole chromosomes that are unable to travel to the spindle poles during mitosis. Since this method has a good reliability and reproducibility it is a standard mutagenic test system for the detection of genetic toxicology [FENECH, 2000; FENECH, 2007].

Nucleoplasmic bridges and nucleoplasmic buds (see Figure 5) provide an additional and complementary measure of chromosome rearrangement that can be scored together with the micronucleus count [FENECH, 2000].

With the CBMN assay it is possible to obtain the frequency of chromosome breaks and chromosome loss with MNi as biomarker. Measurements of chromosome rearrangements like dicentric chromosomes (nucleoplasmatic bridges (NPBs) as biomarker for DNA misrepair) or gene amplifications (nuclear buds (NBUDs) as biomarker) as well as necrosis and apoptosis are possible [FENECH, 2007].

Bonassi and co-workers for example found that an increased frequency of micronuclei in peripheral blood lymphocytes can probably predict cancer risk in humans, especially urogenital and gastro-intestinal cancer [BONASSI et al., 2007].

The principle of this assay is that cells are stimulated for division by PHA and after an addition of cytochalasin B, a mycotoxin for blocking cytokinesis the cell growth is stopped at a binucleated state. If there are chromosomal damage micronuclei, NPBs, NBUDs are formed. The cell suspension is paced on slides and after staining the cells are analysed with a microscope in order to count the existing DNA damage products. [FENECH, 2000]
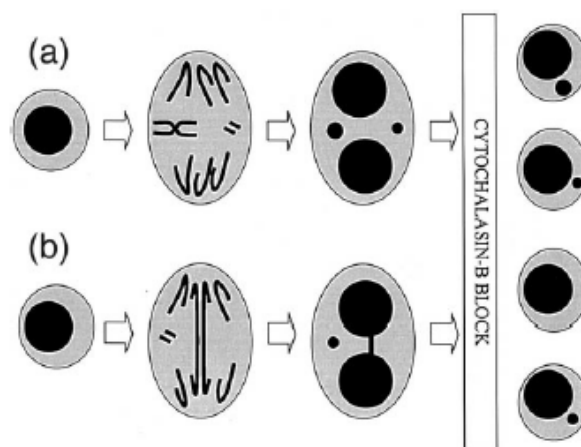
Figure 5: Visualization of micronuclei assay: [a] Micronuclei in anaphase (whole chromosomes and chromosome fragments); [b] Formation of nucleoplasmic bridges (NPBs); [FENECH, 2000]

2.2.4. 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 tail that is build 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 pit and measures the different intensities of the formed "comets" (see Figure 6).

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]
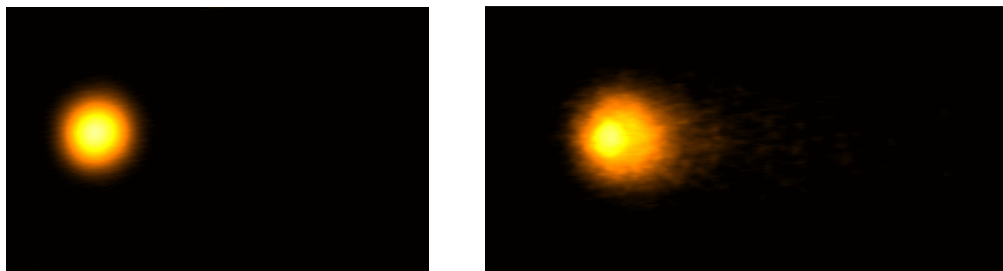
Figure 6: Photomicrographs of DNA from human lymphocytes after electrophoresis (left: no "comet": no DNA migration; right: "comet": damaged DNA, after treatment with ENDOIII)

By using specific enzymes it is possible to make the assay more specific and sensitive since these enzymes can recognize a particular kind of damage. Endonuclease III (ENDOIII) can be used to detect oxidized pyrimidines and formaminopyrimidine DNA glycosylase (FPG) to detect the major purine oxidation product 8-oxoguanine (8-OxoG) as well as other altered purines [Collins, 2004].

After treatment with H_2O_2 mainly DNA strand breaks are occurring but also some oxidation in bases can be detected [COLLINS, 2009]. The receiving amount of DNA breaks is then characteristic for the changes in the sensitivity of the cells towards exogenous oxidative DNA damage [HOELZL et al., 2009].

2.3. Bile pigments

2.3.1. Bilirubin structure

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 7). [VITEK & 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 'neutralized'. [MC DONAGH 2010a]
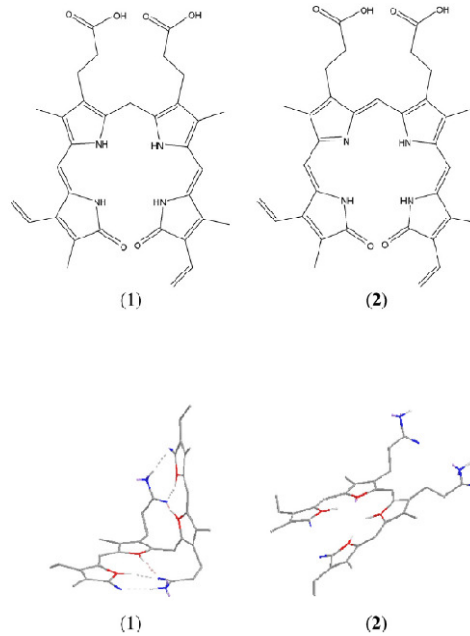
Figure 7: Two- and three-dimensional structures of (1) bilirubin and (2) biliverdin [BULMER et al., 2008b]

2.3.2. Heme Metabolism

Bilirubin, a colourful endogenous compound, is entirely derived from the breakdown of heme in the body.

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 & 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 &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 8) [VITEK & OSTROW 2009].
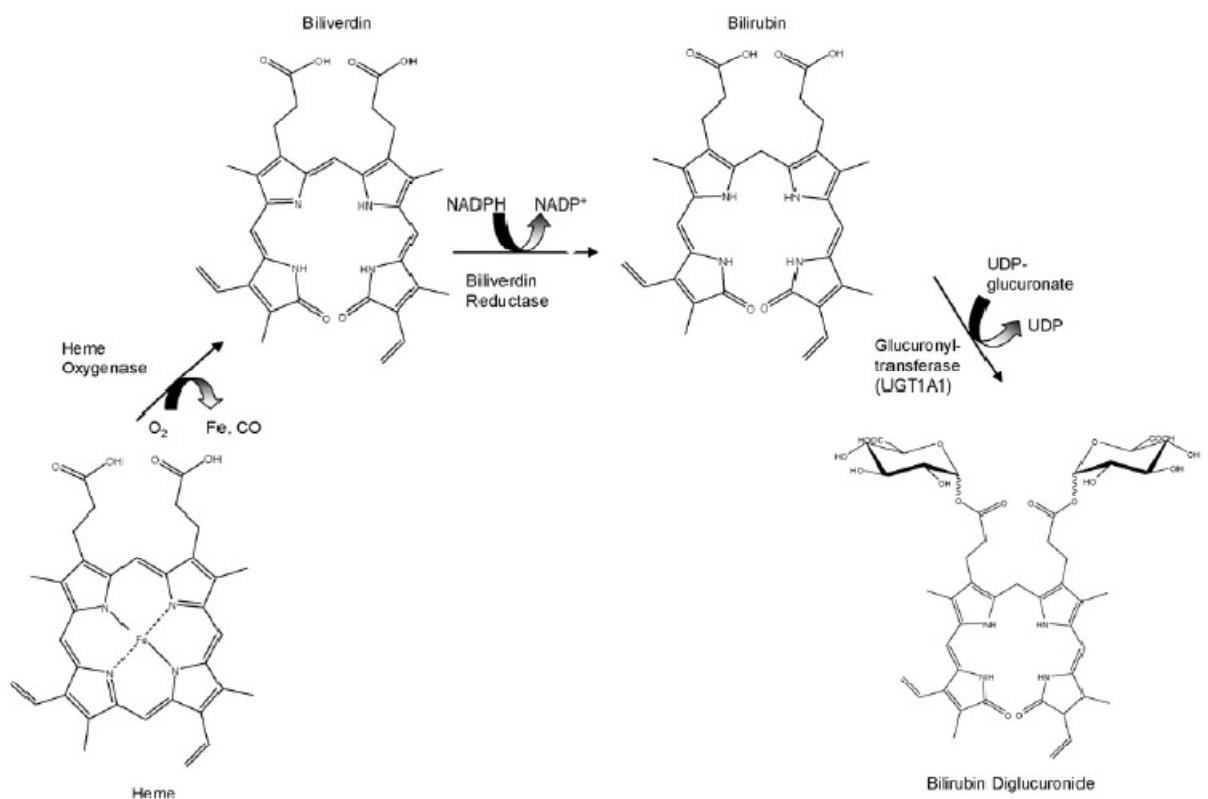
Figure 8: Metabolism of heme [BULMER et al., 2008b]

The first step of heme cleavage which is also the rate limiting step is mediated by HO. Heme oxygenase oxidatively cleaves the heme ring at the methin bridge between pyrrolring A and B 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 to unconjugated bilirubin (UCB). [VITEK & OSTROW, 2009]

With the glucuronyl-transferase (UGT1A1) bilirubin can be conjugated to bilirubin diglucuronide. If this enzyme is not working properly it can lead to an increase in bilirubin levels and bilirubin associated diseases like Gilbert's syndrome, crigler-najjar syndrome or neonatal hyperbilirubinaemia. (See also chapter 2.4.). [PETRIDES, 2007]

Biliverdin is only a transient intermediate. It is entirely and quickly reduced by biliverdin reductase, which 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]

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 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). In the ER unconjugated bilirubin is converted into its water soluble form "direct bilirubin" by conjugation. Bilirubin is conjugated with glucuronic acid on sugar molecules by UDP glucuronosyl transferase (UGT1A1) forming bilirubin monoglucuronide and diglucuronides [KAMISAKO, 2000; MC DONAGH, 2010a].

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].

2.3.3. Physiological concentrations of bilirubin

The physiological concentration of bilirubin in plasma is about 0.1 – 1.2 mg/dl (1.7 – 20.5 µmol/l) [PETRIDES, 2007]. Serum 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. Hyperbilirubinaemia

As already discussed bilirubin that is generated during the decomposition of erythrocytes is called unconjugated bilirubin. It is immediately bound to albumin in blood and transported to 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 hyperbilirubinaemia. [THOMPSON, 2003]

Hyperbilirubinaemia 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 µmol/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 hyperbilirubinaemia (jaundice): conjugated and unconjugated bilirubin levels can be increased [THOMPSON, 2003]

- Crigler-Najjar-Syndrome: >335 µmol/l unconjugated bilirubin [BULMER et al., 2008a]

- Dubin-Johnson Syndrome: 51 – 257 µmol/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". Men are four times more often affected than women [GARTUNG and MATERN, 1999]. It is the most common hereditary cause of increased bilirubin and is found in up to 3 – 17 % of the population [BULMER et al., 2008a].

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].

The condition is characterized by a hereditary mutation in the gene promoter for hepatic uridine diphosphate glucuronosyltransferase (UGT1A1) [BULMER et al., 2008a]. As mentioned before UGT1A1 is responsible for glucuronidation of UCB. In persons with GS a 30 % remaining activity of the enzyme is found. Therefore, the circulating unconjugated bilirubin concentration is increasing. [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 at 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 hyperbilirubinaemia

Neonatal hyperbilirubinaemia 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 µmol/l. In about 70 % of newborns this condition exists but bilirubin levels decline to a normal value after some days and therefore neonatal hyperbilirubinaemia 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 kernicertus can be for example 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 hyperbilirubinaemia, 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 µmol/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. But for some people with CNS reduced enzyme production is not the only cause and for these patients Phenobarbital therapy wouldn't provide any benefits.
[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 µmol/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 hyperbilirubinaemia without cholestasis [THOMPSON, 2003].

In the Dubin-Johnson Syndrome bilirubin levels are between 51 – 257 µmol/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

As already mentioned elevated bilirubin levels that exceed the liver capacity for the excretion can cause toxicity and acute or neurological dysfunction. In fact these patients rarely suffer from bilirubin toxicity and scientists have found that bilirubin acts dose dependent and therefore, might also have a protective role. [BULMER et al., 2008b]

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.

The antioxidative effects of bilirubin were already demonstrated in the 1950s, where it was shown that small quantities of bilirubin or biliverdin can stabilize vitamin A or β-carotene and effectively protect lipids, especially linoleic acid from oxidation [BERNHARD et al., 1954].

In the same experiments it was shown that unconjugated bilirubin reacts efficiently with peroxyl radicals in homogenous solutions. Furthermore a

correlation with membrane-bound α-tocopherol was found concluding that bilirubin and biliverdin may act in synergism with α-tocopherol. In the absence of α-tocopherol lipid peroxidation was increased and with just small amounts of bilirubin or biliverdin this oxidation was inhibited. Stocker and colleagues illustrated that the antioxidant capacity of bilirubin exceeds the one of Vitamin E that is meant to be the strongest antioxidant toward lipid peroxidation so far. [STOCKER and PETERHANS, 1989]

The synergistic effect was also proven in a further study. Bilirubin inhibited the formation of cholesterylester hydroperoxide (CE-OOH), the oxidation product of LDL, by scavenging the alpha tocopherol radical (TO) in LDL. [NEUZIL and STOCKER, 1994]

Additionally other findings showed the idea of bilirubin reacting with ROS. A reason why bilirubin can react with reactive oxygen species is that it contains a pair of reactive hydrogen atoms and acts therefore as H-donator. [STOCKER, 2004]

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

It is assumed that the antioxidant effect of bilirubin originates from the redox-recycling with biliverdin. As already mentioned bilirubin is oxidised to biliverdin and then recycled by biliverdin reductase to bilirubin [BARANANO et al., 2002; McDonagh, 2010b]. In the study of Baranano and co-workers especially the formation of biliverdin during 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) oxidation of bilirubin is ment to be the basic principle of this theory. [BARANANO et al., 2002]

This biliverdin-bilirubin recycling mechanism explaining for the antioxidative protection is becoming more accepted. [VITEK and OSTROW, 2009]

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 prove the antioxidant capacity of bile pigments in vitro but in fact 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 hyperbilirubinaemic Gunn rats were exposed to a hyperoxia (>95 % O_2) atmosphere for 3 days. Oxidative markers were inversely correlated to serum bilirubin concentrations. [DENNERY et al., 1995]

Another important work demonstrating 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 for cardiovascular diseases

Bulmer et al. (2007) investigated bilirubin as protector for cardiovascular diseases in patients with GS. The study was demonstrating that people with GS have improved resistance to serum oxidation and thus a reduced prevalence of cardiovascular disease compared to a control group. In conclusion it was suggested that higher levels of bilirubin can increase circulating antioxidant status and improve resistance to serum oxidation and may protect from prevalence of developing cardio vascular 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 just in general 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 which 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 µM of bilirubin was 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 µM 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 recent 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]

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. 16 persons could not be allocated to one of the groups clearly due to their UCB level very close to 1 mg/dl. There was a difference between first and second blood sampling in reaching threshold values. For a clear allocating 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 summarized in Figure 9.
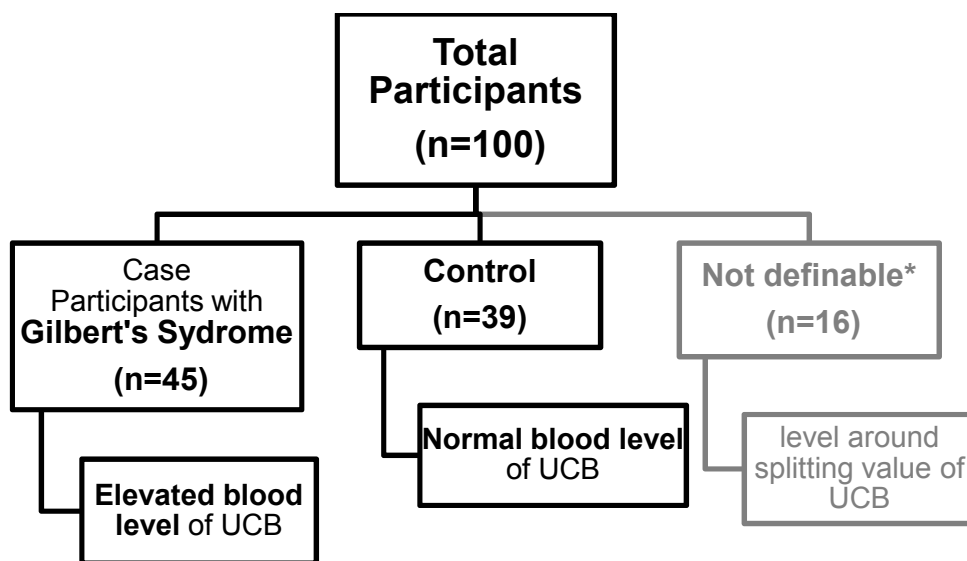
Figure 9: Illustration of the experimental design

* The group "Not definable" is explained particularly in chapter 3.4.

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). Together with questionnaires the inclusion and exclusion criteria were checked.

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

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

Before the comet assay was carried out cells had to be isolated from whole blood and counted. See chapter 3.1.1 and chapter 3.1.2.

Inclusion criteria:

- Age: 20-80 years
- total bilirubin level > 1.2 mg/dl [20.52 μmol/l] (GS)
- unconjugated bilirubin level > 1.0 mg/dl [17.1 μmol/l] (GS)
- total bilirubin level <1.2 mg/dl [20.52 μmol/l] (Control)
- unconjugated bilirubin level <1.0 mg/dl [17.1 μmol/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
- Chronically kidney illness
- 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 pipetted 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 done 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 $1x\ 10^6$ cells/ml is necessary for the comet assay procedure and a total suspension volume of 250 µl. For cell count determination we used the automated cell counter "Countess" from Invitrogen.

A cell suspension of appropriate concentration was needed to be prepared (approx. 10^6 cells/ml) and therefore 20 µl of cell suspension was diluted in 60 µl

PBS-buffer in a cup. 10 µl cell suspension was mixed with 10 µl 0.4 %Trypan blue (1:1). Finally, 10 µl 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 pyrimidenes (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

Material	Company	Product number
Analytical balance	Mettler	AT201
Balance	Mechatronics Austria GmbH	41107690
Calibration solution (pH buffer solution ±0.01pH)	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 Germany, Hitachi	
Microscope slides	VWR-Supplier Partnership for Customer Solutions	ECN 631-1551
Microscope Cover glasses (22x22 mm, 20x20 mm)	VWR	ECN 631-1570
Microwave	Elta GmbH Germany	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)		

Table 2:   Equipment for Comet Assay

3.3.2. Reagents for Comet Assay

Reagents	Abbreviation	Company	Product no.
Albumin from bovine serum	BSA	SIGMA Life science	A2153-50G
Dulbecco´s Phosphate Buffered Saline	PBS	SIGMA Life science	D5837
Ethylenediaminetetraacetic acid	EDTA	SIGMA Life science	
Ethidium bromide aqueous solution		SIGMA Life science	E1510
Hydrochloric Acid	HCl	Riedel-de Haen	
HEPES		SIGMA Life science	

Hydrogen peroxide solution 30 wt % solution in water	H_2O_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		FLUKA Analytica	93350
Triton X-100		SIGMA-Aldrich	
Trypan blue stain 0.4%		INVITROGEN	T10282
Ultra Pure Agarose	NMA	INVITROGEN	0000153001
Ultra Pure LMP Agarose	LMA	INVITROGEN	0000144556

Table 3: Reagents for Comet Assay

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.

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 H_2O. 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 H_2O 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).

H_2O_2 Stock solution

103 µl Hydrogen peroxide solution (30%)

Double distilled H_2O

103 µl of the hydrogen peroxide solution was mixed with 10 ml double distilled H_2O 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 H_2O

Triton X-100

NaCl, EDTA and Tris were dissolved in 1 l double distilled H_2O 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 just before use and then the solution was stored at 4 °C in the cooling chamber.

NaOH (10N)

400 g NaOH

Double distilled H_2O

400 g NaOH were dissolved in 1 l double distilled H_2O to receive a 10N NaOH solution. Stored at room temperature.

Neutralization buffer (pH = 7.5)

48.44 g Tris base (0.4M)

Double distilled H_2O

48.44 g Tris base (0.4M) were dissolved in 1 l double distilled H_2O 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 H_2O

1000 mg NMA were dissolved in 100 ml double distilled H_2O, 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 H_2O

20 µl of ethidium bromide solution was mixed with 10 ml double distilled H_2O 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 10.
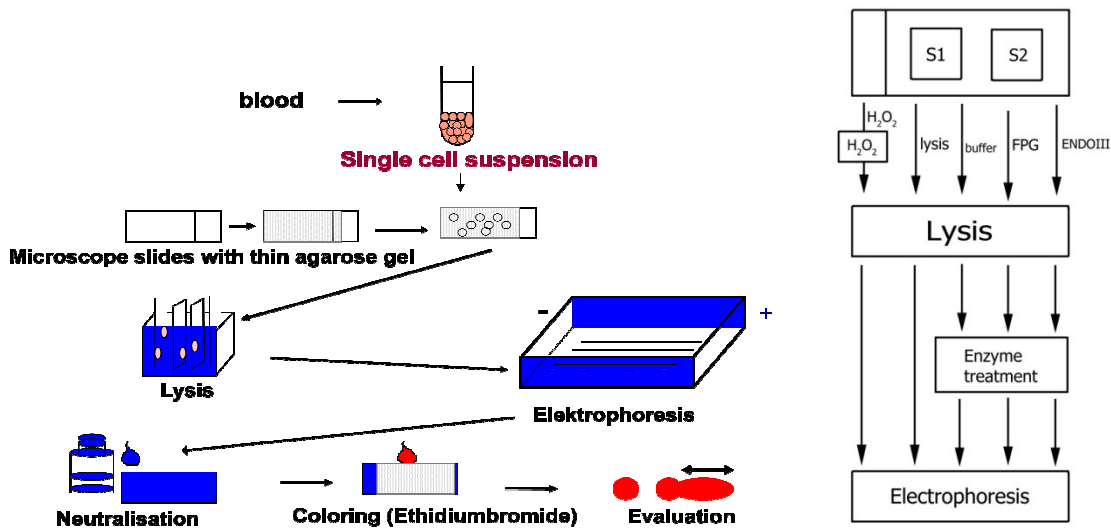
Figure 10:   General activity of comet assay (left) and specific gels treatment (right)

For lysis, FPG, ENDOIII and buffer 3 gels were prepared and 2 gels for H_2O_2. Each slide contained two gels and 2 subjects (S1, S2) were mixed, in general 14 slides were prepared for 2 participants.

Slides treated with lysis stayed in lysis solution all the time, whereas H_2O_2 slides were first treated with hydroperoxide and then kept in lysis solution until electrophoresis. Slides treated with buffer, FPG or ENDOIII stayed in lysis solution (at least 7 minutes) until treatment with enzymes started. See also Figure 10.

Slide preparation:

- Prepared NMA was heated 3 times in microwave until it was completely liquefied 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 and air dried, before they were stored at room temperature.

Embedding cells in agarose: (see Figure 11)

- Prepared LMA was heated 3 times in microwave until it was completely liquefied and then kept warm at 37 °C in a water bath.

- 30 µl of cell suspension (with a concentration of c= $1x10^6$ cells per ml) was resuspended in a cup with 140 µl LMA.

- 70 µl of this mixture were pipetted on a precoated slide, which was placed on cooled trays. On each slide there was place for two spots with a volume of 70 µl.

- Each slide was 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.

Treatment with H_2O_2:

- After the cover slips have been removed from the slides, they were placed in a staining jar containing a cooled H_2O_2 solution and incubated for 5 minutes in a cooling chamber (4 °C).

- Then the slides were washed 3 times in PBS.

Lysis:

- After H_2O_2 incubation and removing the cover slips from all other slides, they were placed in the prepared lysis solution (lysis solution with 1 % triton X) for 75 minutes in the cooling chamber.

- Slides without H_2O_2 incubation were incubated separately from slides with H_2O_2 incubation.

Enzyme treatment:

- Enzyme reaction buffer was prepared.

- Slides for enzyme incubation 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.

- All other slides (H_2O_2 and "no treatment") stayed meanwhile in the lysis solution.

Alkaline treatment and electrophoresis: (see Figure 12)

- 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 µl of the ethidium bromide solution (20 µg/ml) was placed on the slide and covered with a coverslip (22x22 mm).

- For examination two out of three slides were selected and always 50 cells per gel were counted. In case values of the two slides differed too much the third one was additionally determined.
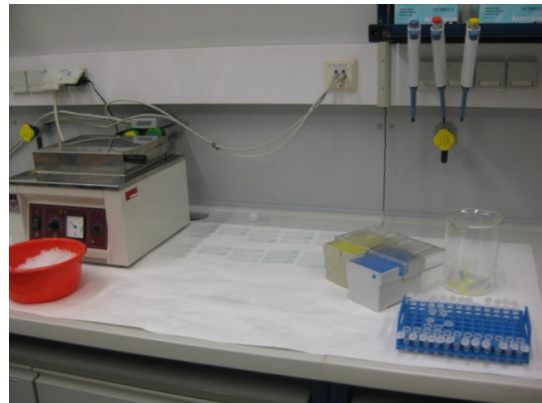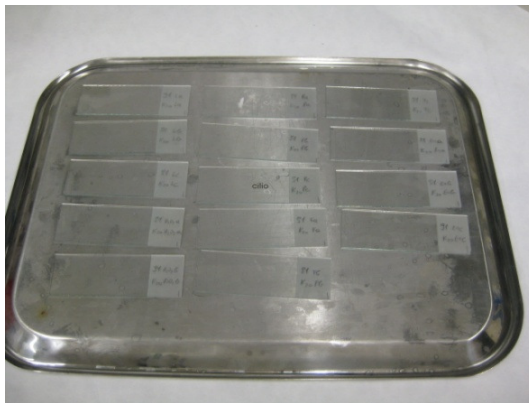
Figure 11:   **Slides on the tray before start of embedding cells in agarose.**
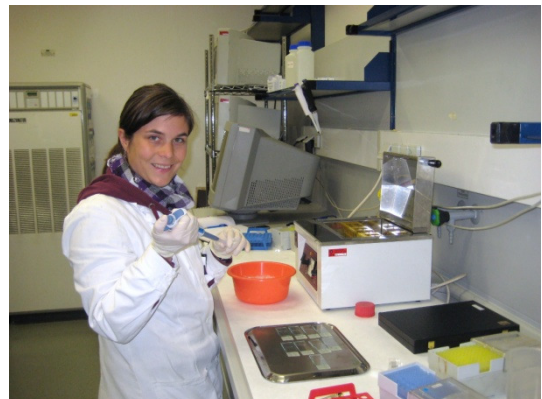
Figure 12:   **Electrophoresis (left) and lab work (right)**

3.4. Statistically Analysis

All statistical analysis was done with SPSS Statistics 17.0. Data was proved with 1-sample Kolmogorow-Smirnow-Test (KS-Test) for normal distribution.

Boxplots were performed on all data in order to identify extreme values.

Mean was compared using "Independent sample t-Test", when comparing 2 groups (Gilbert's Syndrome and Control) and ANOVA-test (Tukey-HSD) was performed, when comparing more than 2 groups (UCB-levels in 3 groups).

Correlations were tested with bivariate "Pearson Correlation" and linear regression was analysed stepwise. All results are expressed as mean values ± standard deviation.

If differences were significant ($p<0.05$) they are marked with [*]. If they were highly significant ($p<0.01$) they are marked with [**]. Results that were very high significant ($p<0.001$) are marked with [***].

As already mentioned bilirubin levels (mean UCB levels) were divided in groups, mentioned as clear defined "Gilbert's Syndrome" (UCB levels >1 mg/dl; [17.1 µmol/l]), "Control" (UCB levels <1 mg/dl; [17.1 µmol/l]) and "Not definable" (bilirubin levels around 1 mg/dl; [17.1 µmol/l]). In the group "Not definable" people with bilirubin levels around 17.1 µmol/l are included. Two measurements were performed and time values of UCB were once over and once under the threshold. Therefore, the group "Not definable" was not included in our statistics when GS and C group were compared. But bilirubin levels were also divided in three groups for statistically analysis, so they are mentioned as group 1 (low bilirubin: 0.24-0.65 mg/dl; [4.1-11.1 µmol/l]), group 2 (moderate bilirubin: 0.66-1.21 mg/dl; [11.3-20.7 µmol/l]) and group 3 (high bilirubin: 1.22-4.3 mg/dl; [20.9-73.5 µmol/l]) (with "not definable-group" included, n=16).

Data was also interpreted stratified after gender and age. Age groups were defined as younger group (<30 year old test subjects) and older group (≥30 year old test subjects).

In the present diploma thesis results of lysis and H_2O_2 are presented. Results of the enzymes FPG and EndoIII treated samples are in the thesis of Nadja Antl "Effects of moderate hyperbilirubinaemia on DNA damage".

4. Results and discussion

Within the present study we wanted to examine, whether serum bilirubin has an antigenotoxic potential. Furthermore, it was an aim to figure out if people with higher bilirubin levels (GS) are better protected against oxidative stress and therefore have less DNA damage than control subjects.

In the present study the very first Comet Assay data in Gilbert's Syndrome is shown but there is no comparable literature available so far.

Descriptive statistics:

	Gilbert's Syndrome	Control	Not definable	Total sample size
Men	35	23	12	70
Women	10	16	4	30
Total	45	39	16	100

Table 4:   Descriptive Statistics of the test subjects

	Mean Age ± SD	Mean UCB levels ± SD (mg/dl)	p
Total Subjects	31.6 ± 11.79	1.2 ± 0.89	
Gilbert's Syndrome	33.2 ± 13.04	1.9 ± 0.83***	<0.001
Control	32.3 ± 11.60	0.5 ± 0.19***	
Men	31.1 ± 11.71	1.4 ± 0.94**	<0.01
Women	32.8 ± 12.09	0.8 ± 0.62**	

Table 5:   Comparison of mean age and mean UCB levels in the different groups

4.1. DNA damage

4.1.1. Gilbert's and Control Group

In the following chapters 4.1.1, 4.2.1 and 4.3.1 unconjugated serum bilirubin data are separated in two groups defined as Gilbert's Syndrome and control group.

All test subjects		N	Mean ± SD (% DNA in tail)	p
Lysis	Gilbert's Syndrome	45	5.3±1.35	n.s.
	Control	37	5.0±1.19	
H_2O_2	Gilbert's Syndrome	45	19.6±4.91	n.s.
	Control	32	18.4±4.24	

Table 6: % DNA in tail in Gilbert's (GS) and control (C) group (all test subjects)

There were no significant differences between Gilbert's and control group in single and double strand breaks.
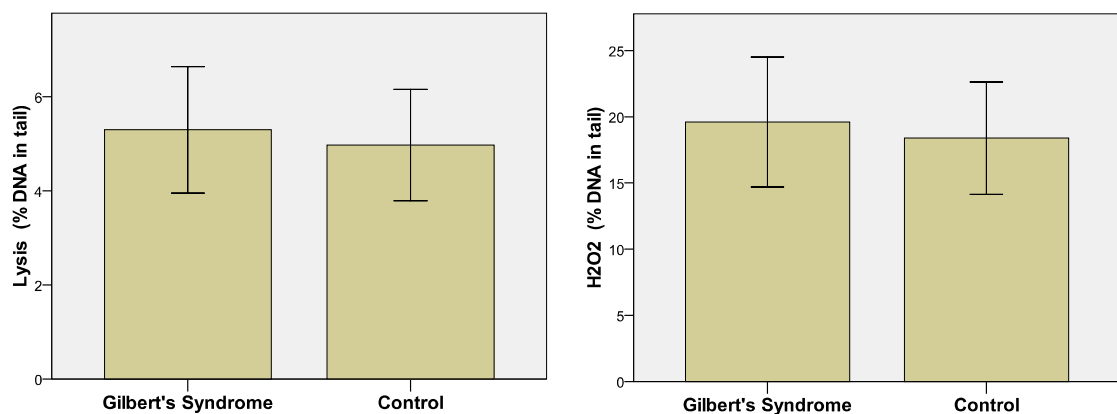
Figure 13:   % DNA in tail in GS and C group (all test subjects)

4.1.2. UCB (unconjugated bilirubin) in 3 Groups

Further we divided unconjugated serum bilirubin data in 3 groups:

➤ group 1 (low bilirubin): 0.24-0.65 mg/dl

➤ group 2 (moderate bilirubin): 0.66-1.21 mg/dl

➤ groups 3 (high bilirubin): 1.22-4.3 mg/dl

In the following chapters 4.1.2, 4.2.2 and 4.3.2 data was interpreted concerning these groups.

All test subjects		N	Mean ± SD (% DNA in tail)
Lysis	Low bilirubin (1)	31	4.9±1.21
	Moderate bilirubin (2)	34	5.3±1.15
	High bilirubin (3)	33	5.1±1.33
H$_2$O$_2$	Low bilirubin (1)	27	18.5±4.65
	Moderate bilirubin (2)	33	18.9±4.33
	High bilirubin (3)	33	19.0±5.01

Table 7: DNA damage in all test subjects according to their bilirubin levels

In all test subjects there were no significant differences observed in mean DNA damage concerning the 3 different bilirubin groups.
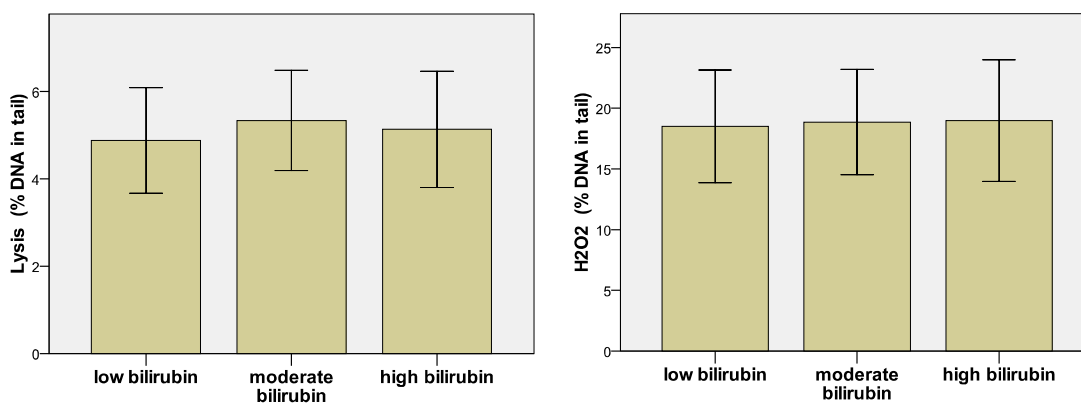
Figure 14:   DNA damage in all test subjects according to their bilirubin levels

4.1.3. Correlation of DNA Damage with Serum UCB

<30 years		Lysis	H_2O_2		≥30 years		Lysis	H_2O_2
Lysis	r	1	0.209		Lysis	r	1	0.389[*]
	p		n.s.			p		<0.05
H_2O_2	r	0.209	1		H_2O_2	r	0.389[*]	1
	p	n.s.				p	<0.05	
UCB	r	-0.020	**-0.126**		**UCB**	r	0.124	**0.369**[*]
	p	n.s.	**n.s.**			p	n.s.	**<0.05**

Table 8:   Correlation of Comet Assay data with UCB regarding age (all test subjects)
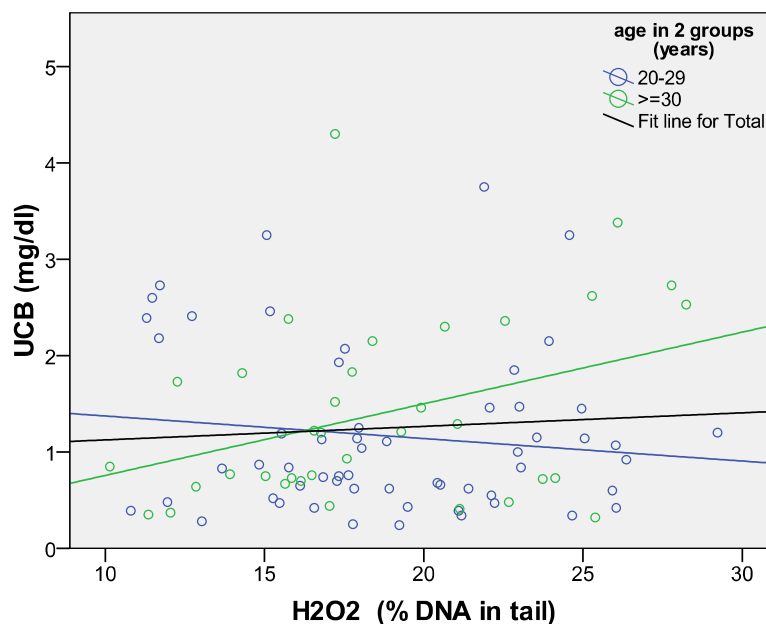
Figure 15:   Correlation of H₂O₂ and UCB regarding age (all test subjects)

Correlation between H_2O_2 and bilirubin in general was not significant. As you can see in Figure 15 the older group showed a positive correlation **(r=0.369; p<0.05)** between UCB and H_2O_2 induced DNA damage.

≥30 year old men		Lysis	H_2O_2	UCB
Lysis	r	1	0.334	0.215
	p		n.s.	n.s.
H_2O_2	r	0.334	1	0.583[**]
	p	n.s.		<0.01

Table 9:   Correlation of Comet Assay data with UCB (≥30 year old men)
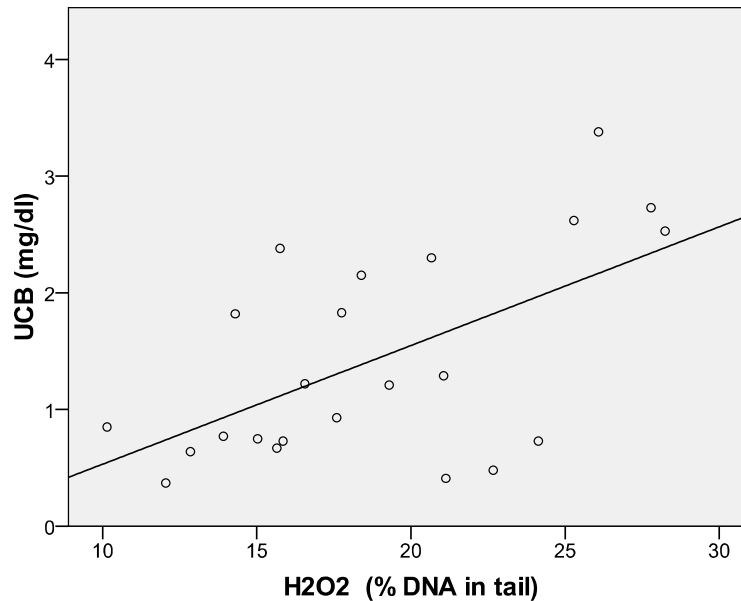
Figure 16:   Correlation of H_2O_2 and UCB (≥30 year old men)

The correlation was even stronger when only considering ≥30 year old male subjects for H_2O_2 and UCB levels (r=0.583; p<0.01).

Since bilirubin has antioxidative potential our hypothesis was to find a negative correlation of bilirubin and DNA damage.

In fact we found a strongly significant positive correlation in the ≥30 year old male subjects. Possible confounding factors like environmental factors, eating habits, or in general lifestyle of test subjects could have influenced the results.

With increasing age higher levels of oxidative stress occur in the body and at the same time antioxidants decrease [SOHAL and WEINDRUCH, 1996]. Further male gender is a risk factor for genetic damage [LAM et al., 2002]. Apart from the gender study participants had an average age of about 31.6 years. And in fact younger people are less affected by oxidative stress and might have a better immune system. Oxidative damage to DNA increases with the age, because cells lose more and more the ability to repair damaged DNA [HAMILTON et. al., 2001]. Fenech also observed in 1998 that micronuclei frequencies increase with the age and DNA damage is more often observed in older people than in younger test subjects [FENECH, 1998]. So this could also have influenced our study results.

In our study you could also see that trends between young and old people were different. In the people older than 30 years a stronger but negative effect on DNA damage after stressing with H_2O_2 was shown. So bilirubin couldn't protect DNA of oxidative damage due to H_2O_2. In young people UCB showed no negative trend on DNA.

4.2. DNA damage: Stratified for gender

4.2.1. Gilbert's and Control Group

Men and Women		sex	N	Mean ± SD (% DNA in tail)
Gilbert's Syndrome	Lysis	men	35	5.5±1.27
		women	10	4.5±1.36
	H_2O_2	men	35	19.8±5.06
		women	10	19.0±4.54
Control	Lysis	men	22	5.1±1.23
		women	15	4.7±1.12
	H_2O_2	men	19	18.6±4.09
		women	13	18.1±4.60

Table 10: % DNA in tail in GS and C group regarding sex

The mean value of DNA damage both in lysis and H_2O_2 showed no significance between men and women. In the control group the mean for men was 8.5 % higher for lysis and 2.8 % higher for H_2O_2 than for women. In the GS group the mean in men was 22.2 % higher for lysis and 4.2 % higher for H_2O_2 than in women. But in general the average mean of % DNA in tail in men was not significantly higher than in women.
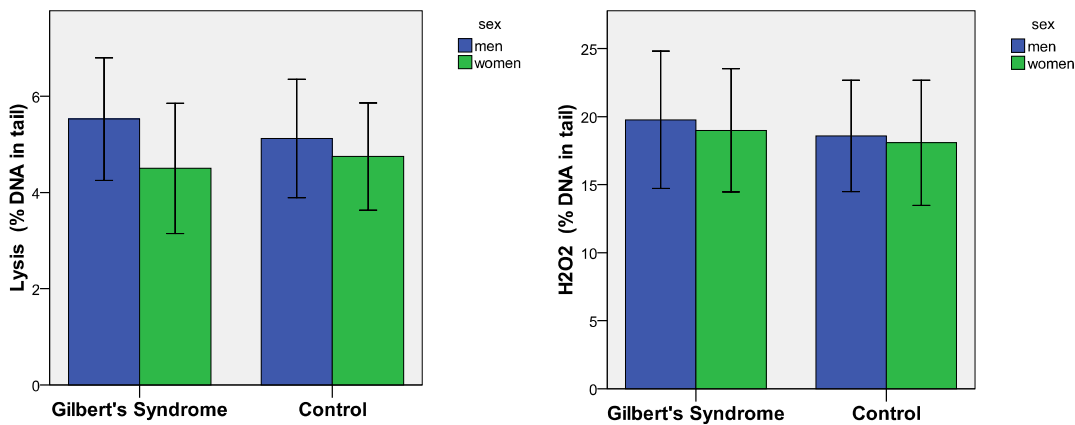
Figure 17:   % DNA in tail in GS and C group regarding sex

In fact there was no significant mean difference in lysis or H_2O_2 in all male test subjects as well as in all female test subjects.

Lysis data for women with GS were 18.2 % lower than for men and in the control group women had 7.8 % lower DNA damage.

In H_2O_2 results of lysis were similar as women with GS had 4 % lower mean values than men and in the control group 2.7 %.

Concluding the results, men showed slightly higher DNA damage than women, without being significant. This result can be also confirmed by the study of Lam et al., who observed that male gender was associated as a risk factor for genetic damage [LAM et al., 2002].

Although in our study men were slightly less resistant towards oxidation by H_2O_2 which was confirmed earlier by Bajpayee et al (2002), another study by Dusinska and Collins showed the opposite in 2008. This study showed that men tend to be more resistant towards H_2O_2 stress than women [BAJPAYEE et al., 2002; DUSINSKA and COLLINS, 2008].

4.2.2. UCB in 3 Groups

	Men	N	Mean ± SD (% DNA in tail)
Lysis	Low bilirubin (1)	16	5.3±1.32
	Moderate bilirubin (2)	26	5.5±1.13
	High bilirubin (3)	27	5.3±1.26
H_2O_2	Low bilirubin (1)	14	18.4±4.31
	Moderate bilirubin (2)	25	18.7±4.52
	High bilirubin (3)	27	19.2±5.13

Table 11: DNA damage in men according to their bilirubin levels

No significant difference in all groups for lysis and H_2O_2 was observed.

	Women	N	Mean ± SD (% DNA in tail)
Lysis	Low bilirubin (1)	15	4.5±0.95
	Moderate bilirubin (2)	8	4.9±1.19
	High bilirubin (3)	6	4.5±1.58
H_2O_2	Low bilirubin (1)	13	18.6±5.16
	Moderate bilirubin (2)	8	19.5±3.87
	High bilirubin (3)	6	17.9±4.67

Table 12: DNA damage in women according to their bilirubin levels

In women there was no tendency for DNA damage in the different bilirubin groups in lysis and in H_2O_2. All results were not significant.

4.3. DNA damage: Stratified for age

4.3.1. Gilbert's and Control Group

< 30 and ≥ 30		age in 2 groups (years)	N	Mean ± SD (% DNA in tail)
Gilbert's Syndrome	Lysis	<30	26	5.2±1.35
		≥30	19	5.4±1.37
	H_2O_2	<30	26	19.3±5.22
		≥30	19	20.0±4.55
Control	Lysis	<30	23	4.8±1.14
		≥30	14	5.3±1.21
	H_2O_2	<30	20	18.9±3.94
		≥30	12	17.5±4.75

Table 13: % DNA in tail in GS and C group regarding age (all test subjects)

Divided in age groups there were no significant differences concerning mean DNA damage comparing test subjects <30 years with test subjects ≥30 years.
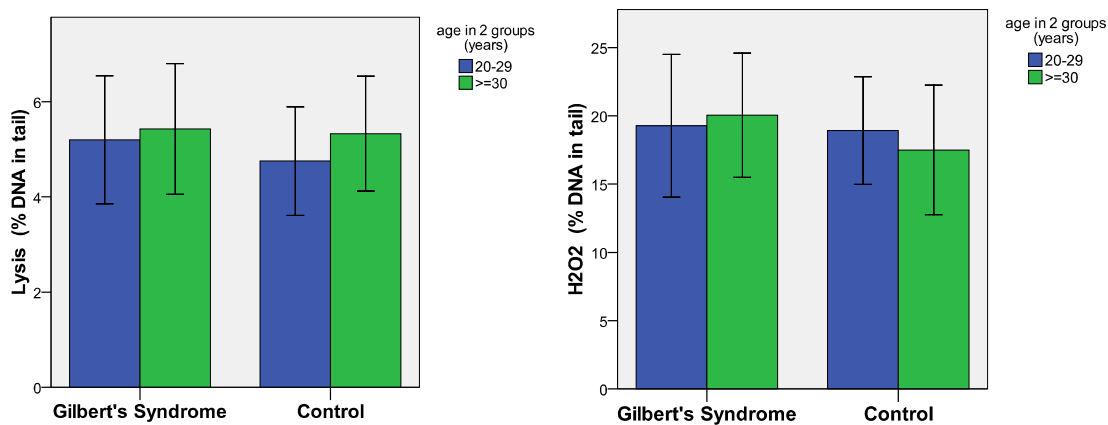
Figure 18:   % DNA in tail in GS and C group regarding age (all test subjects)

In the control group the DNA damage (lysis) was 9.4 % (n.s.) lower in the younger subjects than in older. On the contrary by stressing with H_2O_2 the older groups had lower values (-7.4 %, n.s.).
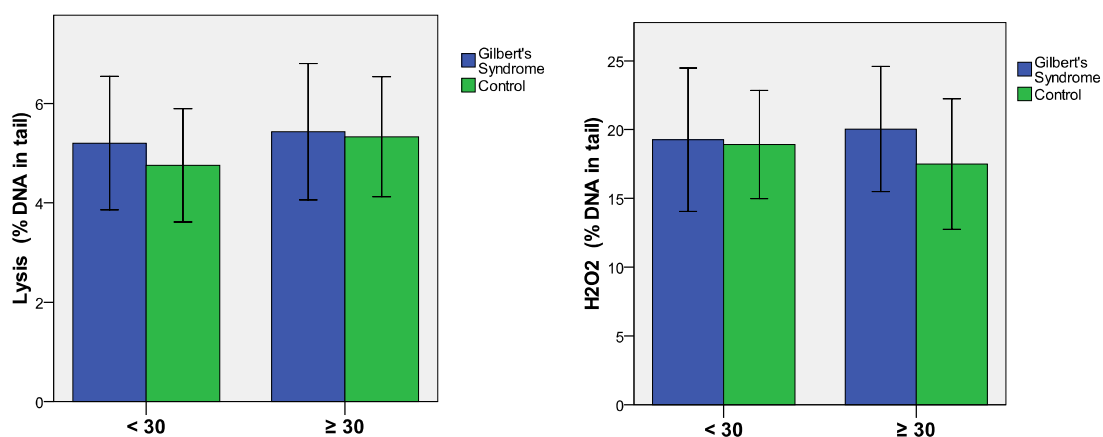
Figure 19: % DNA in tail in age groups regarding GS and C group (all test subjects)

There were no significant differences in lysis and in H_2O_2 challenging in mean DNA damage in all test subjects <30 years.

Same results were found for test subjects ≥30 years.

For lysis in the ≥30 year old subjects there was almost no difference (values for control group were just 1.9 % lower than in Gilbert's group), as on the contrary in the <30 year old test subjects the mean DNA damage was 7.7 % lower in control group than in Gilbert's Syndrome (n.s.).

Furthermore, after H_2O_2 exposure the control group of the ≥30 year old test subjects showed a 12.5 % lower mean DNA damage than Gilbert's Syndrome subjects. On the contrary in the <30 year old test subjects there was no noticeable difference (2.1 %).

The younger GS group showed slightly higher DNA damage after lysis treatment, but on the contrary in the ≥30 year old GS group data for H_2O_2 induced DNA damage was higher, but not significant.

4.3.2. UCB in 3 Groups

< 30 years		N	Mean ± SD (% DNA in tail)
Lysis	Low bilirubin (1)	23	4.6±1.04
	Moderate bilirubin (2)	21	5.6±1.10
	High bilirubin (3)	17	4.8±1.29
H_2O_2	Low bilirubin (1)	20	18.9±4.36
	Moderate bilirubin (2)	21	19.9±4.32
	High bilirubin (3)	17	18.0±5.12

Table 14: DNA damage in <30 year old according to their bilirubin levels; Lysis 1 < 2 (p<0.05)
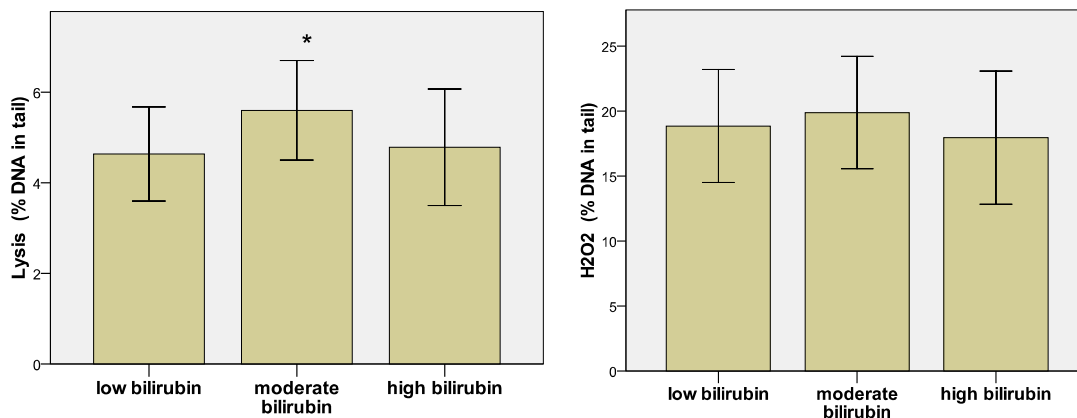
Figure 20:   DNA damage in <30 year old according to their bilirubin levels; Lysis 1 < 2 (p<0.05)

After lysis a significant difference (p<0.05) in mean DNA damage was shown between the "low" and "moderate" bilirubin groups in the <30 year old. The difference was 17.9 %. Another difference between "moderate" and "high" bilirubin groups was "visible" but not significant. The DNA damage in the "high" bilirubin group was 14.3 % lower than in the "moderate" bilirubin group. As shown in Figure 20 the difference between "low" and "high" bilirubin groups was rather small.

Results for H_2O_2 were similar to lysis but not significant.

>30 years		N	Mean ± SD (% DNA in tail)
Lysis	Low bilirubin (1)	8	5.6±1.45
	Moderate bilirubin (2)	13	4.9±1.14
	High bilirubin (3)	16	5.5±1.31
H₂O₂	Low bilirubin (1)	7	17.5±5.65
	Moderate bilirubin (2)	12	17.1±3.89
	High bilirubin (3)	16	20.1±4.81

Table 15: DNA damage in ≥30 year old according to their bilirubin levels
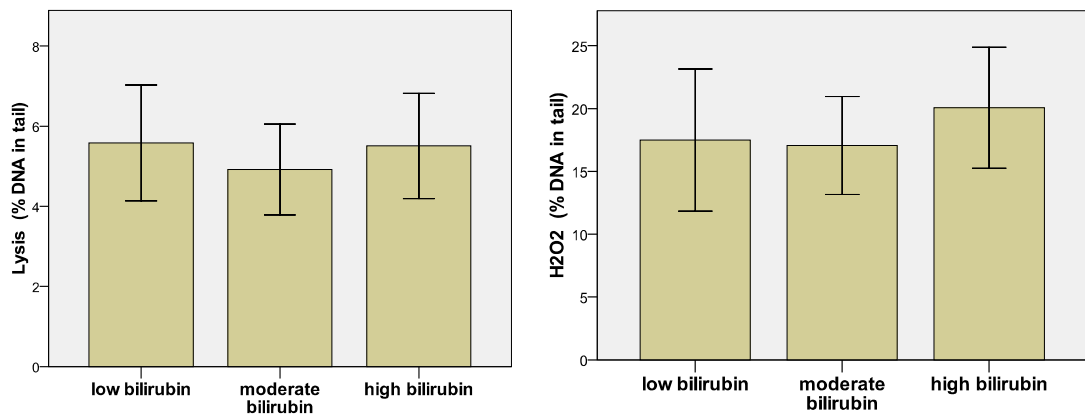
Figure 21:   DNA damage in ≥30 year old according to their bilirubin levels

There were no significant differences in mean DNA damage in test subjects ≥30 years in lysis as well as in H_2O_2 however the mean DNA damage after stressing with H_2O_2 was 14.9 % lower in the "moderate" and "low" bilirubin group comparing to the "high" bilirubin group.

4.3.3. Correlation of DNA damage with age

All test subjects		Lysis	H_2O_2	Age
Lysis	r	1	0.278[**]	0.077
	p		<0.01	n.s.
H_2O_2	r	0.278[**]	1	0.038
	p	<0.01		n.s.

Table 16: Correlation of Comet Assay data with age (all test subjects)

There was no significant correlation of DNA damage with age neither in lysis nor in H_2O_2.

Nevertheless there was a small difference in men and women after lysis. Only in women there was a trend that the DNA damage was increasing with the age (men: r=0.005; n.s.; women: r=0.326; n.s.).

As known from other studies oxidative stress is increasing in the age [SOHAL and WEINDRUCH, 1996; KNIGHT, 2000]. Different studies showed an increase of oxidative damage with age due to following three causes: an increase in the generation of reactive oxygen species (ROS), a decrease in antioxidative defense and a decrease in the efficiency of repair or removal of damaged molecules. [SOHAL and WEINDRUCH, 1996]

Therefore we expected to find an increase in mean DNA damage concerning the age of the test subjects in our study. Actually we were not able to prove this thesis, because our results were not significant so there was no increase in DNA damage with the age. The main reason might be the young age of the study group. There were only a few subjects (n=10) aged 50 years or older.

In literature we could find some inconsistent information, whereas some studies found a difference concerning age in mean DNA damage others didn't. So we could find confirmation for our results. Neither Pitarque and co-workers nor

Carere et al. (2002) could find any significant differences in the Comet assay or the tail moment concerning age. [PITARQUE et al., 1999; CARERE et al., 2002]

4.4. Correlations

We tested the correlation of lysis and H_2O_2 with unconjugated bilirubin (UCB), age, albumin, uric acid and BMI. Furthermore we made stratification for age groups and gender.

4.4.1. Correlation of DNA damage with Serum Albumin

<30 year old women		Lysis	H_2O_2	Serum Albumin
Lysis	r	1	0.161	**-0.565[*]**
	p		n.s.	**<0.05**
H_2O_2	r	0.161	1	**-0.569[*]**
	p	n.s.		**<0.05**

Table 17: Correlation of Comet Assay data with Serum albumin (<30 year old women)
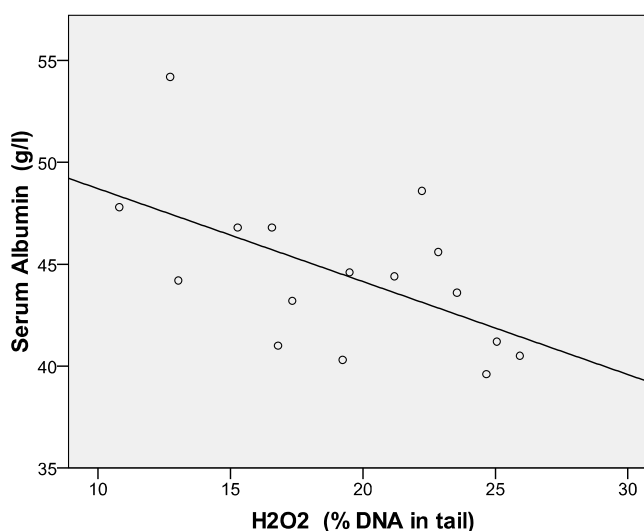
Figure 22:   Correlation of H_2O_2 and Serum albumin (<30 year old women)

There was a highly significant negative correlation between H_2O_2 and albumin. Lower levels of albumins were associated with higher damage in DNA after stressing with H_2O_2. Especially in <30 year old women this correlation was very strong (**r=-0.569; p<0.05**).
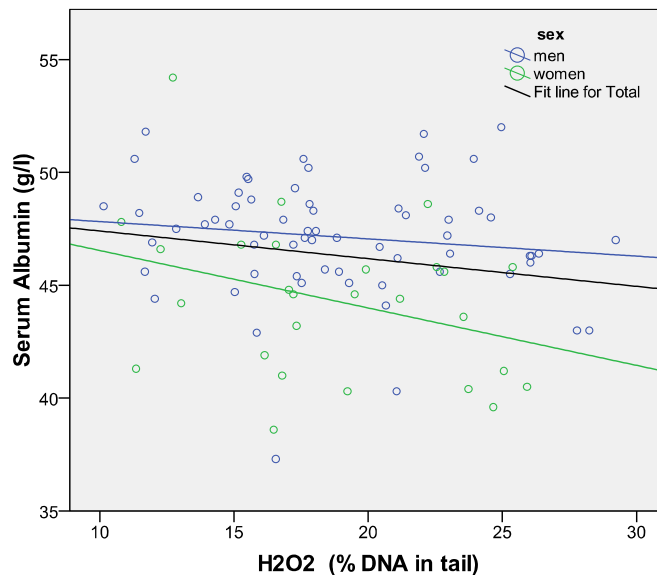
Figure 23: **Correlation of H_2O_2 and Serum albumin regarding sex (all test subjects)**

DNA damage in women (r=-0.336; n.s.) showed a higher correlation with albumin than in men (r=-0.139; n.s.) but results were not significant.

The results for lysis were significant in the younger women with a strong and negative correlation **(r=-0.565; p<0.05)**.

Women		N	Mean ± SD (g/l)
Serum albumin	low bilirubin (1)	16	44.6±2.77
	moderate bilirubin (2)	8	**42.3±3.02**[*]
	high bilirubin (3)	6	**47.1±3.54**[*]
	Total	30	44.5±3.32

Table 18: Mean Serum albumin concentrations according to bilirubin levels in women (moderate UCB < high UCB; p<0.05)

<30 year old		N	Mean ± SD (g/l)
Serum albumin	low bilirubin (1)	23	46.4±3.11
	moderate bilirubin (2)	21	**46.3±2.34**[*]
	high bilirubin (3)	17	**49.0±2.67**[*]
	Total	61	47.1±2.96

Table 19: Mean Serum albumin concentrations according to bilirubin levels in <30 year old subjects (moderate=low UCB < high UCB; p<0.05)

We also observed a significant difference in serum albumin levels in women depending on the bilirubin plasma levels. Subjects <30 years of age showed higher albumin levels in the high bilirubin group compared to the moderate (p<0.01) and low bilirubin group (p<0.05). (see Table 19)

Since albumin is the transporter of bilirubin in blood, elevated levels of bilirubin are linked to elevated levels of serum albumin.

Albumin is the most important plasma protein and represents the first line in defense against reactive oxygen species (ROS) [CANDIANO et al., 2009].

Albumin contains 585 amino acids and the regular plasma concentration in humans is between 35 and 50 g/l. Beside the transport of metals, fatty acids, cholesterol, bile pigments and drugs an important function is the regulation of the osmotic pressure and as important antioxidant in the plasma. In a pro-inflammation state increased albumin concentrations could be found in order to exert its multiple antioxidant properties. [ROCHE et al., 2008]

Previous mentioned bilirubin is bound to albumin in plasma and as studies already have tested albumin-bound bilirubin successfully competes with uric acid for peroxyl radicals [STOCKER at al., 1987].

Free copper and iron can interact with hydrogen peroxide but bound to protein these metals are less susceptible for this reaction. Therefore high concentrations of circulating albumin can reduce the formation of hydroxyl radicals that are products of Fe-H_2O_2 interactions. [ROCHE et al., 2008]

Our study showed that after stressing PBMCs with H_2O_2 the DNA damage was higher at increasing albumin levels. This outcome was basically observed in women.

In our study we could prove the hypothesis that albumin but especially albumin-bound bilirubin, can act as antioxidant and can protect DNA from oxidation by H_2O_2.

4.4.2. Correlation of DNA damage with Uric Acid

All test subjects		Lysis	H_2O_2	Uric acid
Lysis	r	1	0.278[**]	**0.426[***]**
	p		<0.01	**<0.001**
H_2O_2	r	0.278[**]	1	0.010
	p	<0.01		n.s.

Table 20: Correlation of Comet Assay data with Uric acid (all test subjects)

Men		Lysis	H_2O_2		Women		Lysis	H_2O_2
Lysis	r	1	0.290[*]		Lysis	r	1	0.280
	p		<0.05			p		n.s.
H_2O_2	r	0.290[*]	1		H_2O_2	r	0.280	1
	p	<0.05				p	n.s.	
Uric Acid	r	**0.411[***]**	0.012		Uric Acid	r	**0.108**	-0.017
	p	**<0.001**	n.s.			p	**n.s.**	n.s.

Table 21: Correlation of Comet Assay data with Uric Acid regarding sex (all test subjects)
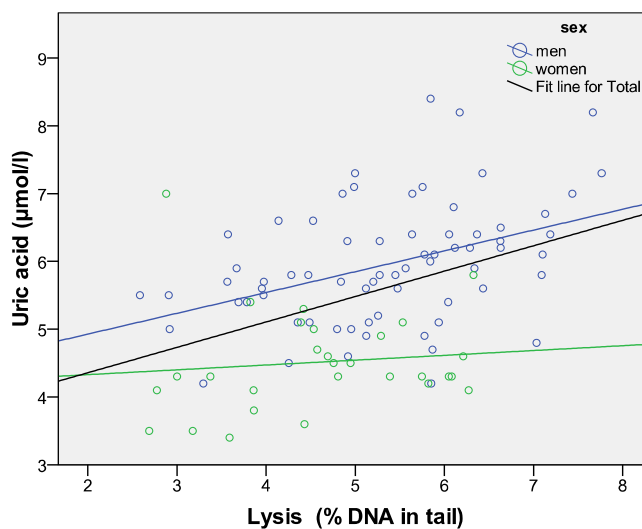
Figure 24: Correlation of Lysis and Uric acid regarding sex (all test subjects)

<30 years		Lysis	H_2O_2		≥30 years		Lysis	H_2O_2
Lysis	r	1	0.209		Lysis	r	1	0.389[*]
	p		n.s.			p		<0.05
H_2O_2	r	0.209	1		H_2O_2	r	0.389[*]	1
	p	n.s.				p	<0.05	
Uric acid	r	**0.533**[***]	-0.122		Uric acid	r	**0.273**	0.238
	p	**<0.001**	n.s.			p	**n.s.**	n.s.

Table 22: Correlation of Comet Assay data with Uric acid regarding age (all test subjects)
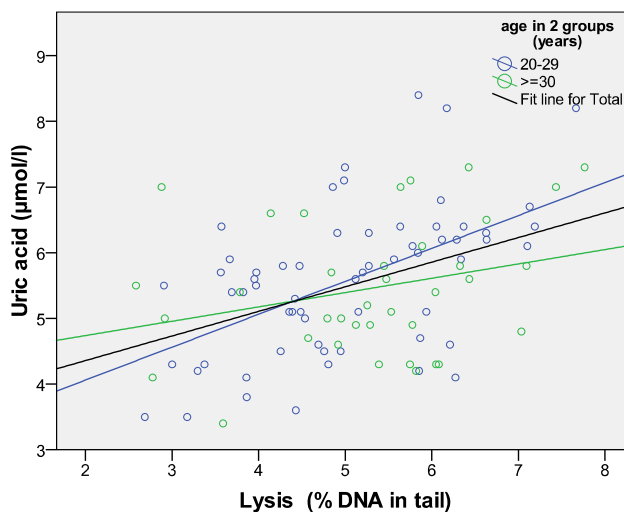
Figure 25: Correlation of Lysis and Uric acid regarding age (all test subjects)

There was a strong positive correlation (r=0.426; p<0.001) between lysis and uric acid that was highly significant, showing that with higher levels of uric acid in blood the damage in DNA in lysis was increasing. This correlation was strong in men, but not seen in women (men: r=0.411, p<0.001; women: r=0.108, n.s.). Particularly the highest correlation was found in younger people (<30 years: r=0.533; p<0.001; ≥30 years: r=0.273; n.s.).

We couldn't find any significant correlations of uric acid with DNA damage after H_2O_2 challenge.

In humans uric acid ($C_5H_4N_4O_3$) is the final breakdown product of purine nucleotides. Uric acid cannot be metabolised and is therefore excreted in urine. Elevated uric acid levels can be harmful and lead to gout. [HORN et al., 2005] In most other mammals, uric acid is oxidised by the enzyme uricase to allantoin. If this enzyme is not working at all higher primates lose the ability to synthesize ascorbic acid too. Both uric acid and ascorbic acid are strong electron donors and can therefore act as antioxidants. In humans uric acid is responsible for over half of the antioxidant capacity in blood plasma. [MAXWELL et al., 1997]

However, uric acid is a double-edged sword. On one hand serum uric acid is a potent antioxidant that even might protect against cancer [KOLONEL et al., 1994] and serve as biggest contributor to serum ferric reducing antioxidant power (FRAP) values [LEE et al., 2008]. On the other hand it is known that it is a strong predictor of cardiovascular disease mortality [NISKANEN et al., 2011]. It is also mentioned that uric acid may be an indicator for increased oxidative stress since xanthine oxidase, an enzyme in the degradation circle of purines to uric acid, has been shown to be an important source of superoxide free radicals [CULLETON et al., 1999]. (See also Figure 26).

In cancer patients abnormal high levels of serum uric acid are often found as a result of increased nucleic acid turnover in the rapidly proliferating diseased tissue [KOLONEL at al., 1994].
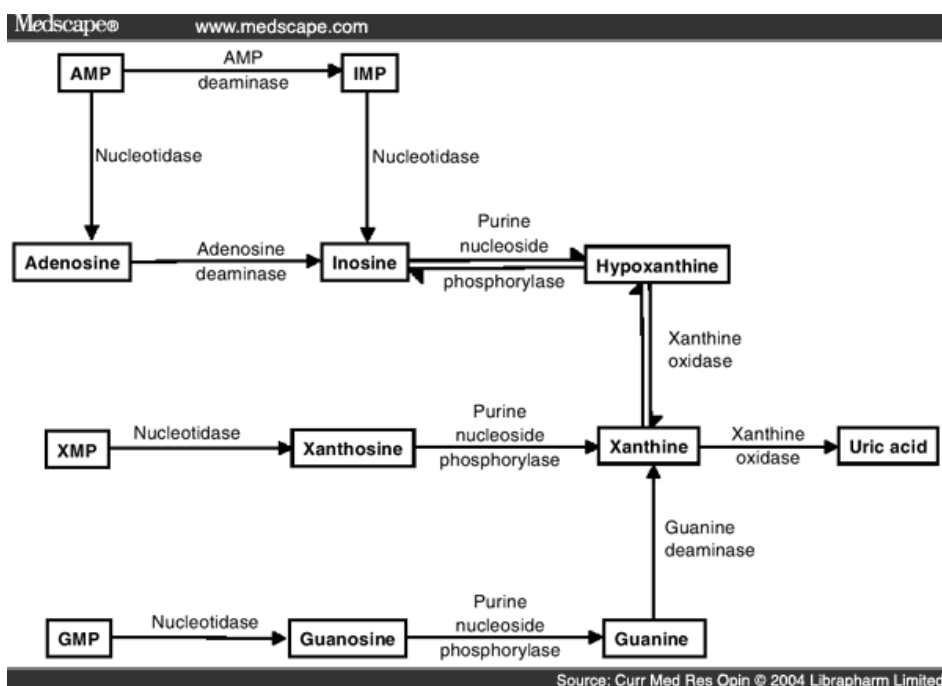
Figure 26: **Purine metabolism from Adenosine to Uric acid [www.medscape.com; access date: 29.4.2011]**

Uric acid	N	Mean ± SD (µmol/l)	p
Total	100	5.5±1.08	
Men	70	6.0±0.90[***]	<0.001
Women	30	4.6±0.74[***]	

Table 23: Mean values of Uric acid in the test subjects

Our study showed a strong positive correlation between serum uric acid and mean DNA damage. Especially in men this outcome was very strong for lysis data. Further it was shown that men have higher uric acid levels which might also explain the higher DNA damage in men. (men: 6.0 µmol/l; women: 4.6 µmol/l)

4.4.3. Correlation of DNA damage with BMI
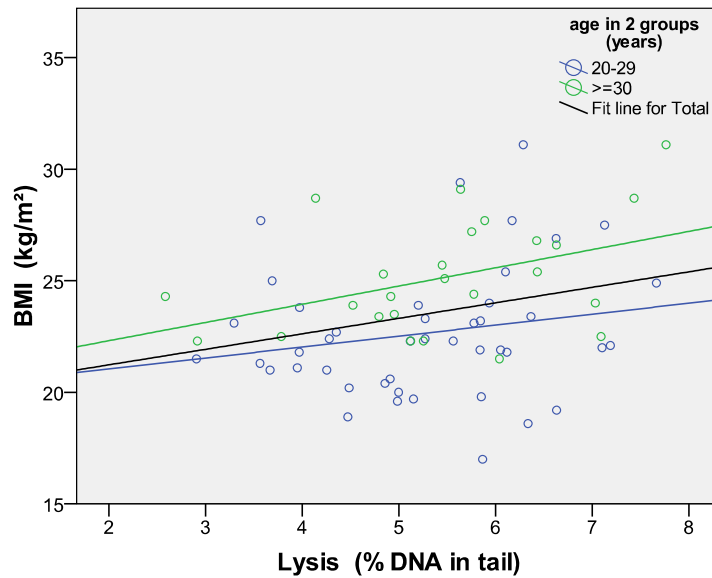
Figure 27: Correlation of Lysis and BMI regarding age (men)

There was a significant positive but weak correlation between mean DNA damage (lysis) and BMI of male subjects (r=0.277; p<0.05). Especially in the ≥30 year old men outcomes were stronger (**r=0.416; p<0.05**) than in the <30 year old men (r=0.195; n.s.).

BMI	N	Mean ± SD (kg/m²)	p
<30 years	61	22.6±2.90**	**<0.01**
≥30 years	39	24.6±3.33**	

Table 24: Mean values of BMI in test subjects regarding age

BMI	N	Mean ± SD (kg/m²)	p
Gilbert's Syndrome	45	22.9±3.00	n.s.
Control	39	24.1±3.31	

Table 25: Mean values of BMI in test subjects regarding GS and C groups

Especially older people might have higher BMI values and have also other BMI classifications. As overweight can lead to oxidative stress BMI is an important factor. In our study test subjects ≥30 years generally had higher BMI levels than subjects <30 years and also mean of BMI was within normal BMI range. Differences were even significant (see Table 24). People with high BMI are more likely to suffer from oxidative stress related diseases like cardiovascular diseases or cancer [SINAIKO et al., 2005]. The higher BMI could be one reason for the negative impact of the older age group on DNA damage.

Comparing the groups Gilbert's syndrome and control there were the same results. Gilbert's syndrome subjects had in general lower BMI levels than controls (see Table 25). Although this better outcome was not significant a trend could be observed (p=0.084).

In conclusion we could find some interesting results regarding the correlation of lysis and H_2O_2 with uric acid, bilirubin, BMI and albumin.

Uric acid levels were higher in men than in women which might explain the higher DNA damage in men that we observed.

Generally serum albumin showed a higher correlation (negative) with DNA damage in women than in men. As already discussed albumin might have a protective role against oxidative stress and this could be a reason why women had less DNA damage than men in our study.

4.5. Linear regression

A linear regression analysis was performed with different variables, but no model was found for H_2O_2 treatment. However for lysis some of the results are shown.

4.5.1. Lysis

	Predictors	Adjusted R²	p	Excluded variables
All test subjects (98)	Uric acid	0.173***	<0.001	
	Uric acid and Albumin	0.226**	<0.01	
Men (69)	Uric acid	0.156***	<0.001	
	Uric acid and Albumin	0.199*	<0.05	
Women (29)	Albumin	0.118*	<0.05	Uric acid
<30 years (61)	Uric acid	0.272***	<0.001	
	Uric acid and Albumin	0.322*	<0.05	
<30 year old men (44)	Uric acid	0.178**	<0.01	Albumin
<30 year old women (17)	Albumin	0.273*	<0.05	Uric acid

Table 26: Linear regression model for DNA damage (Lysis data)

Uric acid could explain 17.3 % of the lysis results and by including albumin 22.6 % could be explained.

For males the variable uric acid showed more influence whereas for females only albumin had a weak relevant impact.

In the <30 year old test subjects uric acid could explain 27.2 % of the lysis results and by including albumin 32.2 %.

In the ≥30 year old test subjects no linear regression could be found neither in male nor in female subjects.

In the <30 year old male subjects 17.8 % of the lysis results could be explained by uric acid, whereas in the <30 year old women almost 30 % could be explained by albumin but for this subgroup total sample size (n=17) was already quite small.

Considering the available data we can conclude that for men uric acid was the more influencing factor of DNA damage whereas for women it was albumin.

5. Conclusion

With this study we wanted to examine, whether serum bilirubin has an antigenotoxic potential. Furthermore, it was an aim to figure out if people with higher bilirubin levels (GS) are better protected against oxidative stress and therefore have less DNA damage than control subjects.

We couldn't confirm our hypothesis because no significant differences in DNA damage between the Gilbert's and the control group were found, as well as there were no significant differences in the 3 bilirubin percentiles. We were not able to find a significant inverse correlation between bilirubin and DNA damage in order to show that higher levels of bilirubin leads to a lower amount of strand breaks in DNA.

But we found a positive correlation between DNA damage and serum uric acid, which means that in our study uric acid acted as oxidant especially in young men.

There was also a negative correlation between DNA damage and serum albumin. We showed that higher albumin levels were associated with lower DNA damage. After stressing with H_2O_2 results were very clear for <30 year old women ($p<0.05$).

We also were able to show that uric acid is a more influencing factor in men whereas for women it is albumin, what also proves our pervious statement.

Another result was that BMI had an influence on DNA damage, especially in the older test subjects. In the groups older than 30 years the higher the BMI levels were the higher was DNA damage.

Furthermore age had no influence on our results.

6. Summary

Many epidemiological studies showed that elevated levels of serum bilirubin have a protective influence on cancer and cardiovascular diseases, since bilirubin acts as antioxidant. Particularly people with Gilbert's Syndrome (GS), a harmless hyperbilirubinaemia, have elevated levels of unconjugated serum bilirubin and therefore might be better protected.

The present case-control-study included 100 test subjects. All participants were randomly assigned to the GS or control group. The study was carried out at the Department of Nutritional Sciences at the University of Vienna. Participants were recruited by information-sheets, inclusion and exclusion criteria were checked by questionnaire.

For measuring DNA damage and resistance to H_2O_2-induced DNA damage the comet assay was applied, which is a well established method for measuring single and double strand breaks.

In fact we were not able to confirm our hypothesis that people with higher bilirubin levels (GS) are more protected against oxidative stress and therefore have less DNA damage than control subjects.

But we found some interesting results on serum albumin and uric acid. Serum albumin worked as antioxidant and showed a more protecting effect on DNA than bilirubin especially in women. On the contrary high levels of uric acid were associated with higher DNA damage. Age had no influence on DNA damage but higher BMI levels correlated with a higher DNA damage, particularly in test subjects older than 30 years of age.

As there is no comparable literature available so far, further studies on people with elevated bilirubin levels are needed to support the hypothesis of the beneficial effects of bilirubin on DNA.

7. Zusammenfassung

Viele epidemiologische Studien zeigten, dass erhöhte Bilirubinspiegel einen schützenden Einfluss auf Krebs und kardiovaskuläre Erkrankungen haben, da Bilirubin als Antioxidans agiert. In Speziellen Personen mit Gilbert Syndrom (GS) - einer harmlosen Hyperbilirubinämie - haben erhöhte Werte von unkonjugiertem Serumbilirubin und sind daher möglicherweise besser geschützt.

In dieser Fall-Kontroll-Studie nahmen 100 Testpersonen teil. Alle Teilnehmer wurden entweder der Gruppe Gilbert Syndrom oder Kontrolle zugeteilt. Die Studie wurde am Institut für Ernährungswissenschaften an der Universität Wien durchgeführt. Die durch Informationszettel angeworbenen Teilnehmer wurden mittels Fragebogen auf Ein- und Ausschlusskriterien überprüft.

Für das Messen von DNA Schäden und der Resistenz gegenüber H_2O_2-induzierten DNA Schäden wurde der „Comet Assay" verwendet, welcher eine weit verbreitete Methode zum Messen von Einzel- und Doppelstrangbrüchen ist.

Wir konnten unsere Hypothese, dass Menschen mit erhöhtem Bilirubinwert (GS) besser vor oxidativem Stress geschützt sind und daher weniger DNA Schäden aufweisen als die Kontrollprobanden, nicht beweisen.

Aber wir konnten einige andere interessante Ergebnisse in Bezug auf Serumalbumin und Harnsäure finden. Serumalbumin wirkte als Antioxidans und zeigte eine höhere Schutzfunktion gegenüber der DNA als Bilirubin, vor allem bei Frauen. Im Gegensatz dazu beobachteten wir, dass hohe Werte von Harnsäure mit erhöhten DNA Schaden einhergingen. Das Alter hatte keinen Einfluss auf DNA Schäden allerdings korrelierte ein höherer BMI mit höheren DNA Schäden, besonders bei Studienteilnehmern älter als 30 Jahre.

Es ist noch keine vergleichbare Literatur vorhanden, daher sind noch mehr Studien mit Personen mit erhöhtem Bilirubinwert notwendig, um die Hypothese, dass Bilirubin einen schützenden Effekt auf die DNA hat, zu prüfen.

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9. Appendix

a. Comparison of Gilbert's and Control Group

a.1. Differences concerning gender

Men	Classification in groups	N	Mean ± SD (% DNA in tail)	p
Lysis	Gilbert's Syndrome	35	5.5±1.27	n.s.
	Control	22	5.1±1.23	
H$_2$O$_2$	Gilbert's Syndrome	35	19.8±5.06	n.s.
	Control	19	18.6±4.09	

Table: Comparison of significant difference in mean in Gilbert's and control group (men)

Women	Classification in groups	N	Mean ± SD (% DNA in tail)	p
Lysis	Gilbert's Syndrome	10	4.5±1.36	n.s.
	Control	15	4.8±1.12	
H$_2$O$_2$	Gilbert's Syndrome	10	19.0±4.54	n.s.
	Control	13	18.1±4.60	

Table: Comparison of significant difference in mean in Gilbert's and control group (women)

a.2. Differences concerning age

<30 years	Classification in groups	N	Mean ± SD (% DNA in tail)	p
Lysis	Gilbert's Syndrome	26	5.2±1.35	n.s.
	Control	23	4.8±1.14	
H$_2$O$_2$	Gilbert's Syndrome	26	19.3±5.22	n.s.
	Control	20	18.9±3.94	

Table: Comparison of significant difference in mean in Gilbert's and control group (<30 years)

≥30 years	Classification in groups	N	Mean ± SD (% DNA in tail)	p
Lysis	Gilbert's Syndrome	19	5.4±1.37	n.s.
	Control	14	5.3±1.21	
H$_2$O$_2$	Gilbert's Syndrome	19	20.0±4.55	n.s.
	Control	12	17.5±4.75	

Table: Comparison of significant difference in mean in Gilbert's and control group (≥30 years)

a.3. Differences concerning gender and age

<30 year old men	Classification in groups	N	Mean ± SD (% DNA in tail)	p
Lysis	Gilbert's Syndrome	22	5.4±1.31	n.s.
	Control	14	5.0±1.13	
H_2O_2	Gilbert's Syndrome	22	19.3±5.28	n.s.
	Control	12	19.1±3.66	

Table: Comparison of significant difference in Gilbert's and control group (<30 year old men)

≥30 year old men	Classification in groups	N	Mean ± SD (% DNA in tail)	p
Lysis	Gilbert's Syndrome	13	5.7±1.23	n.s.
	Control	8	5.4±1.42	
H_2O_2	Gilbert's Syndrome	13	20.6±4.73	n.s.
	Control	7	17.6±4.91	

Table: Comparison of significant difference in Gilbert's and control group (≥30 year old men)

<30 year old women	Classification in groups	N	Mean ± SD (% DNA in tail)	p
Lysis	Gilbert's Syndrome	4	4.1±1.04	n.s.
	Control	9	4.4±1.14	
H_2O_2	Gilbert's Syndrome	4	19.4±5.63	n.s.
	Control	8	18.6±4.57	

Table: Comparison of significant difference in Gilbert's and control group (<30 year old women)

≥30 year old women	Classification in groups	N	Mean ± SD (% DNA in tail)	p
Lysis	Gilbert's Syndrome	6	4.8±1.55	n.s.
	Control	6	5.2±0.98	
H_2O_2	Gilbert's Syndrome	6	18.7±4.22	n.s.
	Control	5	17.3±5.07	

Table: Comparison of significant difference in Gilbert's and control group (≥30 year old women)

b. Comparison of UCB bilirubin in 3 levels

All test subjects	Groups	Groups	p
Lysis	1	2	
	1	3	n.s.
	2	3	
H_2O_2	1	2	
	1	3	n.s.
	2	3	

Table: Comparison of significant difference in mean in three groups of bilirubin (all test subjects)

b.1. Differences concerning gender

Men	Groups	Groups	p
Lysis	1	2	
	1	3	n.s.
	2	3	
H_2O_2	1	2	
	1	3	n.s.
	2	3	

Table: Comparison of significant difference in mean in three levels of bilirubin (men)

Women	Groups	Groups	p
Lysis	1	2	
	1	3	n.s.
	2	3	
H_2O_2	1	2	
	1	3	n.s.
	2	3	

Table: Comparison of significant difference in mean in three levels of bilirubin (women)

b.2. Differences concerning age

< 30 years	Group	Group	p
Lysis	1	2	<0.05
	1	3	n.s.
	2	3	
H_2O_2	1	2	
	1	3	n.s.
	2	3	

Table: Comparison of significant difference in mean in three levels of bilirubin (<30 years)

≥30 years	Groups	Groups	p
Lysis	1	2	
	1	3	n.s.
	2	3	
H_2O_2	1	2	
	1	3	n.s.
	2	3	

Table: Comparison of significant difference in mean in three levels of bilirubin (≥30 years)

c. Correlation

c.1. Correlation between lysis and H_2O_2 with the age, albumin, bilirubin, uric acid, glutathione and creatinine

All test subjects		Lysis	H_2O_2	Age	Albumin	UCB	Uric acid	Glutathione	Creatinine
Lysis	r	1							
	p								
H_2O_2	r	0.278**	1						
	p	<0.01							
Age	r	0.077	0.038	1					
	p	n.s.	n.s.						
Albumin	r	-0.137	-0.182	-0.328**	1				
	p	n.s.	n.s.	<0.01					
UCB	r	0.055	0.073	0.125	0.208*	1			
	p	n.s.	n.s.	n.s.	<0.05				
Uric acid	r	0.426***	0.010	-0.075	0.235*	0.179	1		
	p	<0.001	n.s.	n.s.	<0.05	n.s.			
Glutathione	r	0.225*	0.082	0.149	-0.245*	-0.051	-0.151	1	
	p	<0.05	n.s.	n.s.	<0.05	n.s.	n.s.		
Creatinine	r	0.298**	0.095	-0.033	0.302**	0.129	0.537***	-0.229*	1
	p	<0.01	n.s.	n.s.	<0.01	n.s.	<0.001	<0.05	

Table: Correlation lysis and H_2O_2 with age, albumin, bilirubin, uric acid, glutathione and creatinine (all test subjects)

Men		Lysis	H_2O_2	Age	Albumin	UCB	Uric acid	Glutathione	Creatinine
Lysis	r	1							
	p								
H_2O_2	r	0.290*	1						
	p	<0.05							
Age	r	0.005	0.036	1					
	p	n.s.	n.s.						
Albumin	r	-0.244*	-0.139	-0.517***	1				
	p	<0.05	n.s.	<0.001					
UCB	r	-0.013	0.098	0.153	0.036	1			
	p	n.s.	n.s.	n.s.	n.s.				
Uric acid	r	0.411***	0.012	-0.144	-0.015	0.030	1		
	p	<0.001	n.s.	n.s.	n.s.	n.s.			
Glutathione	r	0.309**	0.138	0.065	-0.242*	-0.019	-0.012	1	
	p	<0.01	n.s.	n.s.	<0.05	n.s.	n.s.		
Creatinine	r	0.106	0.113	-0.013	0.176	-0.070	0.221	-0.051	1
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

Table: Correlation lysis and H_2O_2 with age, albumin, bilirubin, uric acid, glutathione and creatinine (men)

Women		Lysis	H$_2$O$_2$	Age	Albumin	UCB	Uric acid	Glutathione	Creatinine
Lysis	r	1							
	p								
H$_2$O$_2$	r	0.280	1						
	p	n.s.							
Age	r	0.326	0.045	1					
	p	n.s.	n.s.						
Albumin	r	-0.387[*]	-0.336	0.009	1				
	p	<0.05	n.s.	n.s.					
UCB	r	-0.050	-0.020	0.142	0.370[*]	1			
	p	n.s.	n.s.	n.s.	<0.05				
Uric acid	r	0.108	-0.017	0.235	0.005	-0.016	1		
	p	n.s.	n.s.	n.s.	n.s.	n.s.			
Glutathione	r	0.315	-0.034	0.319	-0.058	0.093	-0.048	1	
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
Creatinine	r	0.308	0.165	0.130	-0.332	-0.154	0.042	-0.309	1
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

Table: Correlation lysis and H$_2$O$_2$ with age, albumin, bilirubin, uric acid, glutathione and creatinine (women)

<30 year olds		Lysis	H$_2$O$_2$	Age	Albumin	UCB	Uric acid	Glutathione	Creatinine
Lysis	r	1							
	p								
H$_2$O$_2$	r	0.209	1						
	p	n.s.							
Age	r	-0.014	0.060	1					
	p	n.s.	n.s.						
Albumin	r	-0.072	-0.258	0.050	1				
	p	n.s.	n.s.	n.s.					
UCB	r	-0.020	-0.126	0.124	0.373[**]	1			
	p	n.s.	n.s.	n.s.	<0.01				
Uric acid	r	0.533[***]	-0.122	0.017	0.302[*]	0.212	1		
	p	<0.001	n.s.	n.s.	<0.05	n.s.			
Glutathione	r	0.331[**]	0.190	-0.273[*]	-0.167	0.083	-0.046	1	
	p	<0.01	n.s.	<0.05	n.s.	n.s.	n.s.		
Creatinine	r	0.362[**]	0.076	0.111	0.385[**]	0.186	0.632[***]	-0.234	1
	p	<0.01	n.s.	n.s.	<0.01	n.s.	<0.001	n.s.	

Table: Correlation lysis and H$_2$O$_2$ with age, albumin, bilirubin, uric acid, glutathione and creatinine (<30 year olds)

≥30 year olds		Lysis	H$_2$O$_2$	Age	Albumin	UCB	Uric acid	Glutathione	Creatinine
Lysis	r	1							
	p								
H$_2$O$_2$	r	0.389*	1						
	p	<0.05							
Age	r	-0.044	0.178	1					
	p	n.s.	n.s.						
Albumin	r	-0.158	-0.121	-0.252	1				
	p	n.s.	n.s.	n.s.					
UCB	r	0.124	0.369*	0.083	0.084	1			
	p	n.s.	<0.05	n.s.	n.s.				
Uric acid	r	0.273	0.238	-0.082	0.096	0.149	1		
	p	n.s.	n.s.	n.s.	n.s.	n.s.			
Glutathione	r	0.014	-0.007	-0.337*	-0.104	-0.329*	-0.297	1	
	p	n.s.	n.s.	<0.05	n.s.	<0.05	n.s.		
Creatinine	r	0.211	0.121	-0.006	0.165	0.066	0.371*	-0.233	1
	p	n.s.	n.s.	n.s.	n.s.	n.s.	<0.05	n.s.	

Table: Correlation lysis and H$_2$O$_2$ with age, albumin, bilirubin, uric acid, glutathione and creatinine (≥30 year olds)

<30 year old men		Lysis	H$_2$O$_2$	Age	Albumin	UCB	Uric acid	Glutathione	Creatinine
Lysis	r	1							
	p								
H$_2$O$_2$	r	0.261	1						
	p	n.s.							
Age	r	-0.052	0.064	1					
	p	n.s.	n.s.						
Albumin	r	-0.245	-0.097	0.010	1				
	p	n.s.	n.s.	n.s.					
UCB	r	-0.147	-0.152	0.104	0.253	1			
	p	n.s.	n.s.	n.s.	n.s.				
Uric acid	r	0.444**	-0.130	-0.088	0.047	0.124	1		
	p	<0.01	n.s.	n.s.	n.s.	n.s.			
Glutathione	r	0.488**	0.261	-0.313*	-0.170	0.116	0.112	1	
	p	<0.01	n.s.	<0.05	n.s.	n.s.	n.s.		
Creatinine	r	0.149	0.090	0.113	0.165	0.009	0.352*	-0.172	1
	p	n.s.	n.s.	n.s.	n.s.	n.s.	<0.05	n.s.	

Table: Correlation lysis and H$_2$O$_2$ with age, albumin, bilirubin, uric acid, glutathione and creatinine (<30 year old men)

<30 year old women		Lysis	H₂O₂	Age	Albumin	UCB	Uric acid	Glutathione	Creatinine
Lysis	r	1							
	p								
H₂O₂	r	0.161	1						
	p	n.s.							
age	r	-0.014	0.064	1					
	p	n.s.	n.s.						
Albumin	r	-0.565*	-0.569*	0.021	1				
	p	<0.05	<0.05	n.s.					
UCB	r	-0.098	-0.022	0.131	0.421	1			
	p	n.s.	n.s.	n.s.	n.s.				
Uric acid	r	0.293	-0.183	0.190	-0.384	-0.492*	1		
	p	n.s.	n.s.	n.s.	n.s.	<0.05			
Glutathione	r	0.087	-0.048	-0.081	-0.059	0.164	-0.285	1	
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
Creatinine	r	0.231	0.287	-0.047	-0.228	-0.112	0.160	-0.377	1
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

Table: Correlation lysis and H₂O₂ with age, albumin, bilirubin, uric acid, glutathione and creatinine (<30 year old women)

≥30 year old men		Lysis	H₂O₂	Age	Albumin	UCB	Uric acid	Glutathione	Creatinine
Lysis	r	1							
	p								
H₂O₂	r	0.334	1						
	p	n.s.							
Age	r	-0.110	0.100	1					
	p	n.s.	n.s.						
Albumin	r	-0.233	-0.235	-0.385	1				
	p	n.s.	n.s.	n.s.					
UCB	r	0.147	0.583*	0.141	-0.050	1			
	p	n.s.	<0.05	n.s.	n.s.				
Uric acid	r	0.389	0.255	-0.119	-0.243	-0.086	1		
	p	n.s.	n.s.	n.s.	n.s.	n.s.			
Glutathione	r	-0.007	-0.036	-0.371	-0.051	-0.311	-0.139	1	
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
Creatinine	r	0.028	0.157	-0.044	0.233	-0.190	-0.039	0.180	1
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

Table: Correlation lysis and H₂O₂ with age, albumin, bilirubin, uric acid, glutathione and creatinine (≥30 year old men)

≥30 year old women		Lysis	H₂O₂	Age	Albumin	UCB	Uric acid	Glutathione	Creatinine
Lysis	r	1							
	p								
H₂O₂	r	0.521	1						
	p	n.s.							
Age	r	0.195	0.445	1					
	p	n.s.	n.s.						
Albumin	r	-0.153	0.090	0.141	1				
	p	n.s.	n.s.	n.s.					
UCB	r	-0.136	0.046	-0.037	0.330	1			
	p	n.s.	n.s.	n.s.	n.s.				
Uric acid	r	-0.131	0.204	0.135	0.453	0.313	1		
	p	n.s.	n.s.	n.s.	n.s.	n.s.			
Glutathione	r	0.262	0.130	-0.433	0.014	-0.176	-0.149	1	
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		
Creatinine	r	0.424	-0.047	0.514	-0.514	-0.192	-0.034	-0.380	1
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

Table: Correlation lysis and H_2O_2 with age, albumin, bilirubin, uric acid, glutathione and creatinine (≥30 year old women)

c.2. Correlation between lysis and H_2O_2 with vitamin C, homocysteine, iron, LDL, HDL, cholesterol and BMI

All test subjects		Lysis	H₂O₂	Vitamin C	Homocysteine	Iron	LDL	HDL	CHol	BMI
Lysis	r	1								
	p									
H₂O₂	r	0.278[**]	1							
	p	<0.01								
Vitamin C	r	0.057	0.000	1						
	p	n.s.	n.s.							
Homocysteine	r	-0.010	0.062	0.043	1					
	p	n.s.	n.s.	n.s.						
Iron	r	-0.027	0.046	0.151	-0.051	1				
	p	n.s.	n.s.	n.s.	n.s.					
LDL	r	-0.079	-0.150	0.002	0.053	0.095	1			
	p	n.s.	n.s.	n.s.	n.s.	n.s.				
HDL	r	-0.164	0.062	0.029	0.030	-0.053	-0.063	1		
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.			
CHol	r	-0.118	-0.136	-0.026	0.059	0.060	0.925[***]	0.247[*]	1	
	p	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	<0.05		
BMI	r	0.175	-0.128	-0.299[**]	0.085	-0.227[*]	0.363[***]	-0.240[*]	0.327[**]	1
	p	n.s.	n.s.	<0.01	n.s.	<0.05	<0.001	<0.05	<0.01	

Table: Correlation lysis and H_2O_2 with vitamin C, homocysteine, iron, HDL, LDL, Cholesterol and BMI (all test subjects)

Men		Lysis	H$_2$O$_2$	Vitamin C	Homocysteine	Iron	LDL	HDL	CHol	BMI
Lysis	r	1								
	p									
H$_2$O$_2$	r	0.290[*]	1							
	p	<0.05								
Vitamin C	r	0.118	0.087	1						
	p	n.s.	n.s.							
Homocysteine	r	-0.071	0.146	0.027	1					
	p	n.s.	n.s.	n.s.						
Iron	r	-0.131	0.018	0.080	-0.104	1				
	p	n.s.	n.s.	n.s.	n.s.					
LDL	r	-0.074	-0.170	0.100	0.204	0.074	1			
	p	n.s.	n.s.	n.s.	n.s.	n.s.				
HDL	r	-0.137	-0.041	0.051	0.087	0.098	-0.030	1		
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.			
CHol	r	-0.081	-0.204	0.078	0.239[*]	0.092	0.959[***]	0.167	1	
	p	n.s.	n.s.	n.s.	<0.05	n.s.	<0.001	n.s.		
BMI	r	0.277[*]	-0.033	-0.148	0.205	-0.266[*]	0.348[**]	-0.177	0.351[**]	1
	p	<0.05	n.s.	n.s.	n.s.	<0.05	<0.01	n.s.	<0.01	

Table: Correlation lysis and H$_2$O$_2$ with vitamin C, homocysteine, iron, HDL, LDL, Cholesterol and BMI (men)

Women		Lysis	H$_2$O$_2$	Vitamin C	Homocysteine	Iron	LDL	HDL	CHol	BMI
Lysis	r	1								
	p									
H$_2$O$_2$	r	0.280	1							
	p	n.s.								
Vitamin C	r	0.010	-0.184	1						
	p	n.s.	n.s.							
Homocysteine	r	0.098	-0.132	0.094	1					
	p	n.s.	n.s.	n.s.						
Iron	r	-0.061	0.113	0.384[*]	0.022	1				
	p	n.s.	n.s.	<0.05	n.s.					
LDL	r	-0.245	-0.101	-0.210	-0.485[**]	0.028	1			
	p	n.s.	n.s.	n.s.	<0.01	n.s.				
HDL	r	0.081	0.278	-0.095	0.025	0.033	0.015	1		
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.			
CHol	r	-0.186	0.064	-0.285	-0.465[*]	0.023	0.872[***]	0.428[*]	1	
	p	n.s.	n.s.	n.s.	<0.05	n.s.	<0.001	<0.05		
BMI	r	-0.080	-0.340	-0.538[**]	-0.193	-0.263	0.401[*]	-0.291	0.306	1
	p	n.s.	n.s.	<0.01	n.s.	n.s.	<0.05	n.s.	n.s.	

Table: Correlation lysis and H$_2$O$_2$ with vitamin C, homocysteine, iron, HDL, LDL, Cholesterol and BMI (women)

<30 year olds		Lysis	H_2O_2	Vitamin C	Homocysteine	Iron	LDL	HDL	CHol	BMI
Lysis	r	1								
	p									
H_2O_2	r	0.209	1							
	p	n.s.								
Vitamin C	r	0.185	0.041	1						
	p	n.s.	n.s.							
Homocysteine	r	-0.047	0.143	0.099	1					
	p	n.s.	n.s.	n.s.						
Iron	r	-0.002	0.027	0.195	0.036	1				
	p	n.s.	n.s.	n.s.	n.s.					
LDL	r	-0.119	-0.082	0.194	0.011	0.148	1			
	p	n.s.	n.s.	n.s.	n.s.	n.s.				
HDL	r	-0.229	0.061	-0.040	0.181	-0.157	-0.182	1		
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.			
CHol	r	-0.204	-0.060	0.149	0.062	0.077	0.900[***]	0.180	1	
	p	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	n.s.		
BMI	r	0.141	-0.166	-0.177	0.081	-0.181	0.217	-0.138	0.251	1
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

Table: Correlation lysis and H_2O_2 with vitamin C, homocysteine, iron, HDL, LDL, Cholesterol and BMI (<30 year olds)

≥30 year olds		Lysis	H_2O_2	Vitamin C	Homocysteine	Iron	LDL	HDL	CHol	BMI
Lysis	r	1								
	p									
H_2O_2	r	0.389[*]	1							
	p	<0.05								
Vitamin C	r	-0.096	-0.073	1						
	p	n.s.	n.s.							
Homocysteine	r	-0.052	-0.029	0.013	1					
	p	n.s.	n.s.	n.s.						
Iron	r	-0.050	0.083	0.054	-0.159	1				
	p	n.s.	n.s.	n.s.	n.s.					
LDL	r	-0.102	-0.223	-0.200	-0.051	0.104	1			
	p	n.s.	n.s.	n.s.	n.s.	n.s.				
HDL	r	-0.116	0.081	0.136	-0.246	0.195	0.010	1		
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.			
CHol	r	-0.101	-0.209	-0.184	-0.110	0.147	0.945[***]	0.274	1	
	p	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	n.s.		
BMI	r	0.151	-0.055	-0.438[**]	-0.114	-0.267	0.429[**]	-0.471[**]	0.289	1
	p	n.s.	n.s.	<0.01	n.s.	n.s.	<0.01	<0.01	n.s.	

Table: Correlation lysis and H_2O_2 with vitamin C, homocysteine, iron, HDL, LDL, Cholesterol and BMI (≥30 year olds)

<30 year old men		Lysis	H₂O₂	Vitamin C	Homocysteine	Iron	LDL	HDL	CHol	BMI
Lysis	r	1								
	p									
H₂O₂	r	0.261	1							
	p	n.s.								
Vitamin C	r	0.354*	0.200	1						
	p	<0.05	n.s.							
Homocysteine	r	-0.093	0.253	0.226	1					
	p	n.s.	n.s.	n.s.						
Iron	r	-0.156	-0.013	0.138	0.008	1				
	p	n.s.	n.s.	n.s.	n.s.					
LDL	r	-0.093	-0.067	0.218	0.133	0.229	1			
	p	n.s.	n.s.	n.s.	n.s.	n.s.				
HDL	r	-0.033	-0.011	-0.006	0.232	-0.039	-0.240	1		
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.			
CHol	r	-0.094	-0.093	0.207	0.213	0.253	0.947***	0.007	1	
	p	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	n.s.		
BMI	r	0.195	-0.095	-0.156	0.146	-0.196	0.252	-0.116	0.292	1
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

Table: Correlation lysis and H₂O₂ with vitamin C, homocysteine, iron, HDL, LDL, Cholesterol, BMI (<30 year old men)

<30 year old women		Lysis	H₂O₂	Vitamin C	Homocysteine	Iron	LDL	HDL	CHol	BMI
Lysis	r	1								
	p									
H₂O₂	r	0.161	1							
	p	n.s.								
Vitamin C	r	0.018	-0.336	1						
	p	n.s.	n.s.							
Homocysteine	r	-0.045	-0.090	-0.170	1					
	p	n.s.	n.s.	n.s.						
Iron	r	-0.025	0.193	0.585*	0.026	1				
	p	n.s.	n.s.	<0.05	n.s.					
LDL	r	-0.336	-0.136	0.160	-0.435	-0.196	1			
	p	n.s.	n.s.	n.s.	n.s.	n.s.				
HDL	r	-0.319	0.207	-0.284	0.207	-0.100	-0.043	1		
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.			
CHol	r	-0.405	0.034	-0.086	-0.350	-0.314	0.791***	0.469	1	
	p	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	n.s.		
BMI	r	0.007	-0.359	-0.233	-0.101	-0.177	0.097	-0.214	0.133	1
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

Table: Correlation lysis and H₂O₂ with vitamin C, homocysteine, iron, HDL, LDL, Cholesterol, BMI (<30 year old women)

≥30 year old men		Lysis	H_2O_2	Vitamin C	Homocysteine	Iron	LDL	HDL	CHol	BMI
Lysis	**r**	1								
	p									
H_2O_2	**r**	0.334	1							
	p	n.s.								
Vitamin C	**r**	-0.230	-0.087	1						
	p	n.s.	n.s.							
Homocysteine	**r**	-0.111	0.007	-0.235	1					
	p	n.s.	n.s.	n.s.						
Iron	**r**	-0.044	0.106	-0.076	-0.284	1				
	p	n.s.	n.s.	n.s.	n.s.					
LDL	**r**	-0.113	-0.325	-0.039	0.111	-0.123	1			
	p	n.s.	n.s.	n.s.	n.s.	n.s.				
HDL	**r**	-0.338	-0.091	0.154	-0.166	0.610[**]	0.291	1		
	p	n.s.	n.s.	n.s.	n.s.	<0.01	n.s.			
CHol	**r**	-0.130	-0.353	-0.051	0.095	-0.041	0.972[***]	0.396[*]	1	
	p	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	<0.05		
BMI	**r**	0.416[*]	0.097	-0.137	0.009	-0.345	0.263	-0.417[*]	0.202	1
	p	<0.05	n.s.	n.s.	n.s.	n.s.	n.s.	<0.05	n.s.	

Table: Correlation lysis and H_2O_2 with vitamin C, homocysteine, iron, HDL, LDL, Cholesterol, BMI (≥30 year old men)

≥30 year old women		Lysis	H_2O_2	Vitamin C	Homocysteine	Iron	LDL	HDL	CHol	BMI
Lysis	**r**	1								
	p									
H_2O_2	**r**	0.521	1							
	p	n.s.								
Vitamin C	**r**	0.135	-0.049	1						
	p	n.s.	n.s.							
Homocysteine	**r**	0.058	-0.130	0.547	1					
	p	n.s.	n.s.	n.s.						
Iron	**r**	-0.156	0.026	0.188	-0.037	1				
	p	n.s.	n.s.	n.s.	n.s.					
LDL	**r**	-0.245	-0.042	-0.530	-0.654[*]	0.244	1			
	p	n.s.	n.s.	n.s.	<0.05	n.s.				
HDL	**r**	0.352	0.472	0.154	-0.355	0.158	0.034	1		
	p	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.			
CHol	**r**	-0.089	0.135	-0.430	-0.728[**]	0.318	0.932[***]	0.378	1	
	p	n.s.	n.s.	n.s.	<0.01	n.s.	<0.001	n.s.		
BMI	**r**	-0.241	-0.327	-0.764[**]	-0.401	-0.351	0.601[*]	-0.406	0.399	1
	p	n.s.	n.s.	<0.01	n.s.	n.s.	<0.05	n.s.	n.s.	

Table: Correlation lysis and H_2O_2 with vitamin C, homocysteine, iron, HDL, LDL, Cholesterol, BMI (≥30 year old women)

Curriculum Vitae

Personal Information

Name   Barbara Rittmannsberger

Date of Birth   19.08.1984

Place of Birth   Linz

Citizenship   Austria

Education

Since 10/2004   Study of Nutritional Sciences at the University of Vienna (Main subject: Nutrition and Environment)

09/1995 - 06/2003   Grammar school "Kollegium Petrinum", Linz

09/1990 – 06/1995   Elementary school "St. Magdalena", Linz

Working Experience

12/2009   Internship at the Department of Nutritional Sciences (University of Vienna), Vienna

07/2009   Internship at the IFUM "Labor für Umweltmedizin" (Virology), Vienna

05-07/2009   Collaboration at the „nutritionDay in Europe" and „nutritionDay im AKH", Vienna

07/2008   Internship at AGES "Lebensmitteluntersuchungsanstalt", Linz

01-05/2008   Erasmus-semester at the „University of Kuopio" (Finland)

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 H_2O_2-treated and untreated PBMCs"

und dem zweiten Titel

"Effects of moderate hyperbilirubinaemia 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.

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Unterschrift Unterschrift

Barbara Rittmannsberger (19.08.1984) Nadja Antl (24.04.1986)

Wien, August 2011