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Katharina Marisch

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LIST of ABBREVIATIONS

AKH	Allgemeines Krankenhaus, General Hospital Vienna
Apo	Apoptotic cells
BN	Binucleated cells
CAD	Coronary artery disease
CBMN	Cytokinesis-block micronucleus assay
CO	Carbon monoxide
CV	Coefficient of variation
CVD	Cardiovascular disease
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
GS	Gilbert's syndrome
HO	Heme oxygenase
HPLC	High-performance liquid chromatography
HUMN	HUman MicronNucleus project
LDL	Low density lipoproteins
LOOH	Linoleic acid hydroperoxide
MN	Micronuclei
MONO	Mononucleated cells
MULT	Multinucleated cells
NBUD	Nuclear bud
NDCI	Nuclear division cytotoxicity index
NDI	Nuclear division index
Necro	Necrotic cells
NPB	Nucleoplasmic bridge
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
ROS	Reactive oxygen species

RPMI	Roswell Park Memorial Institute (medium)
SAM	S-adenosyl methionine
UCB	Unconjugated bilirubin

1 INTRODUCTION

The present thesis was funded by the FWF-Austrian Science Fund and part of the project “The physiological relevance of bile pigments. *In vitro* to *in vivo* evidence of antioxidant, anti-mutagenic and anti-carcinogenic potential and their mechanisms of action.” The project was performed at the Department of Nutritional Sciences that belongs to the University of Vienna. Data were collected together with Maria Theresia Gräfin zu Pappenheim, therefore similarities to her thesis can be observed.

Bile pigments are coloring tetrapyrrols which are derived from the liver within the heme catabolism. These components mainly include bilirubin and biliverdin and were thought to be toxic or useless for a long time. However, especially the unconjugated form of bilirubin has come to researchers focus, as it has been reported to have antioxidant properties. Several *in vitro* and *in vivo* studies have been performed until today, investigating these functions. Certain conditions in humans cause moderately elevated unconjugated bilirubin concentrations, namely Gilbert’s syndrome. While high concentrations of bilirubin are regarded as harmful and toxic, conditions like in Gilbert’s syndrome have been suggested to be even benign.

Oxidative stress, an imbalance between oxidants and antioxidants, can cause increased DNA damage, which is a risk factor for cancer and cardiovascular diseases. The cytokinesis-block micronucleus assay is a reliable method for measuring DNA damage in forms of micronuclei, nucleoplasmic bridges and nuclear buds. It has been shown that these DNA damage markers can predict the risk of cancer.

Consequently, the present study was combining these two facts and aimed to investigate the antioxidant activities of bilirubin in humans and their cancer protective effects. The aim of this thesis was, whether unconjugated bilirubin

can protect the DNA, and further prevent cancer. Moreover, it was examined to evaluate, if antioxidants had a protective effect on DNA stability.

For this purpose, individuals with Gilbert's syndrome and matched healthy controls were recruited and their bilirubin concentrations, as well as their DNA damage were analyzed.

Summarizing, the hypothesis was whether people with Gilbert's syndrome have lower DNA damage than controls.

2 LITERATURE REVIEW

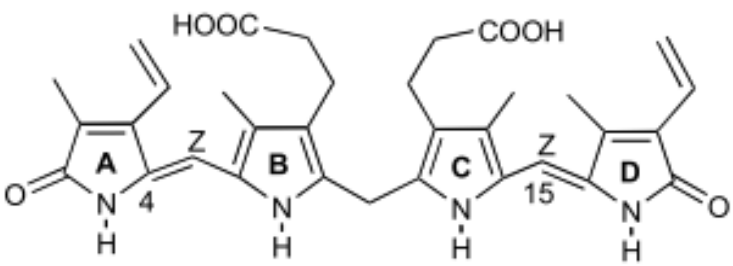
2.1 Bilirubin

Bile or gall is a yellowish-green-brown fluid, produced by the liver and stored in the gallbladder and it helps the body to digest fats. Bilirubin, a bile pigment, is the end product of a physiologic catabolism, occurring only in mammals. In humans, bilirubin appears mainly in the unconjugated form, where bilirubin is not bound to glucuronic acid. To be transported in the body, it is bound to albumin because of its fatsolubility. Bilirubin is unstable, sensitive to oxygen and light and it has been shown to have antioxidant properties [Heirwegh et al., 1989].

2.1.1 Bilirubin chemistry

2.1.1.1 Structure

Bilirubin and biliverdin are colored compounds that belong to a group named bile pigments. These molecules have porphyrine structure, which is composed of four open-chain pyrrolic rings. Bile pigments share the same skeleton but have different ß-substituents, making them specific. Unconjugated bilirubin (UCB) occurs naturally as the IXα-isomer; in 4Z,15Z form. Bilirubin IXα is formed by cleavage of protoporphyrin-IX (heme) at the α-meso-bridge by the enzyme heme oxygenase (HO). Bilirubin has two further isomers, IIIα and XIIIα, which are not detectable in humans [Heirwegh et al., 1989; Vitek & Ostrow, 2009].



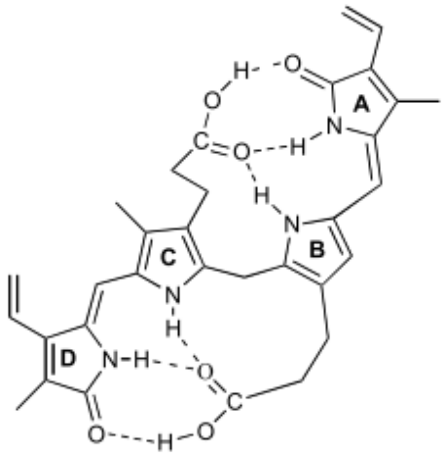


Figure 1. Structure of bilirubin IXα 4Z, 15Z in planar representation and true "ridge-tile" conformation [Vitek & Ostrow, 2009].

2.1.1.2 Solubility

Unconjugated bilirubin is an apolar or lipophilic substance, which is barely soluble in water at physiological pH. However, the presence of two carboxyl groups would indicate water-solubility, but it was shown that these groups are not available for ionization. Bilirubin is almost impossible to dissolve in apolar solvents (e.g. n-hexane, methanol), but in solvents with higher polarity (e.g. chloroform, dichloromethane) its solubility is increasing. Bilirubin dissolves best in dimethyl sulfoxide (DMSO). In body fluids, unconjugated bilirubin is bound to proteins, giving it solubility and making it transportable. In plasma, it is bound to albumin, which has one high affinity binding site for bilirubin. In the cytoplasm, bilirubin is bound to ligandin [Blanckaert, 1981; Brodersen, 1979].

2.1.1.3 Stability and other chemical characteristics

Unconjugated bilirubin is unstable because of the double bonds in the vinyl and methene groups, which can be oxidised easily. On the other side, this ability makes bilirubin a natural antioxidant. Bilirubin is very sensitive to oxygen and light, as well as to high and low pH values [Vitek & Ostrow, 2009].

2.1.1.4 Measurements of bilirubin

Bilirubin is due to the characteristics mentioned above, plus its high affinity for proteins and usually very low concentrations, not easy to determine in tissues and biological fluids. Therefore, many researchers have worked with Gunn rats, a mutant rat with a severe unconjugated hyperbilirubinemia. As bilirubin can be indicative for the diagnosis of different liver diseases, its determination is within clinical interest and several methods have been established (e.g. radioassay, ELISA or immuno-histochemical determination). The simplest determination, though, is the measurement of its absorption via direct absorptiometry (diazo-method). As this method is quite simple and uncomplicated, it is frequently used in hospitals and measured with big analysers, e.g. Vitros. However, this determination is not very sensitive and can only detect high unconjugated bilirubin levels. Thin-layer chromatography was long used for the determination of conjugated bilirubin. At the moment, high-performance liquid chromatography (HPLC) is the preferred method. HPLC determination is highly sensitive and excludes oxygen and light. This analysis is able to detect different bile compounds as well as the bilirubin isomers IIIα, IXα and XIIIα. [Heirwegh et al., 1989; Williams et al., 2006; Zelenka et al., 2008].
Consequently, HPLC was the method of choice in the present thesis project.

2.1.2 Bilirubin metabolism

Bilirubin is formed when hemoglobin is degraded, in a process called heme catabolism. Hemoglobin cracks into heme and globin, heme is further reduced to biliverdin and then to bilirubin. This degradation takes mainly place in the spleen. Bilirubin, which has been reported to be a powerful antioxidant, can also be a potentially toxic compound. Physiologic normal concentrations range up to 17 µmol/L, however, they can be higher and cause certain diseases. This means that the physiological effect is depending on bilirubin concentrations.

2.1.2.1 Heme catabolism

Bilirubin concentrations are related to heme degradation, as bilirubin is the end product of the heme catabolism. Heme is the main source, but also hemoproteins such as hemoglobin, myoglobin, cytochromes and hemoprotein enzymes are included in the pathway. The daily production of bilirubin in humans is approximately 300 mg. Conjugated bilirubin is also called direct bilirubin and unconjugated bilirubin is referred to as indirect. In the total serum, both forms are present, although unconjugated bilirubin is the dominant one in healthy humans [Blanckaert, 1981; Vitek & Schwertner, 2007; Williams et al., 2006].

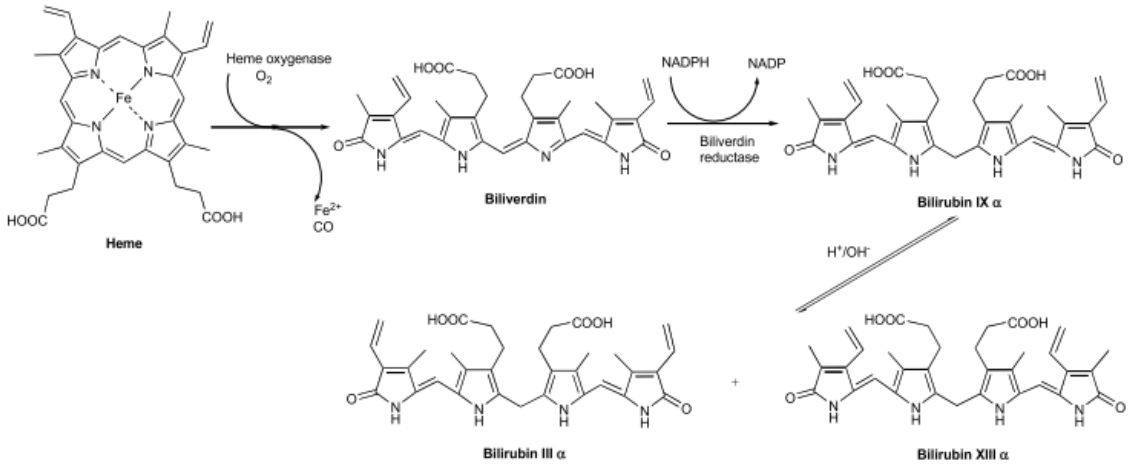


Figure 2. Conversion by heme oxygenase of hemoglobin to biliverdin, CO and Fe^{2+}, with subsequent conversion of biliverdin to unconjugated bilirubin IXα by biliverdin reductase. Shown below are the constitutional IIIα and XIIIα isomers of bilirubin formed by dipyrrole exchange [Vitek & Ostrow, 2009].

The metabolism starts with the breakdown of senescent red blood cells into hemoglobin, occurring mainly in the spleen, but also in the liver and in the bone marrow (reticuloendothelial system). 75% to 80% of the unconjugated bilirubin production arise from hemoglobin, 20% to 25% from non-hemoglobin heme proteins in the liver, immature or defectively formed red cells in the spleen and heme formed in excess of globin in the bone marrow.

Hemoglobin is broken down to heme and globin, and heme is further degraded to biliverdin IXα by the enzyme heme oxygenase, which is the rate-limiting step in this process. Heme oxygenase catalyzes the heme ring by oxidative cleavage of the α-methenyl bridge between two pyrrole rings. At this step, carbon monoxide (CO) and one iron atom are also produced. There are two isoforms of heme oxygenase: HO-1 and HO-2. While HO-1 can be induced by stimuli causing oxidative stress (e.g. free oxygen radicals, heavy metals, bacterial lipopolysaccharides, hydrogen peroxide and ultraviolet light), HO-2 can not be induced.
The enzyme biliverdin reductase reduces biliverdin to bilirubin and is NADPH dependent. Biliverdin reductase has a stereochemical preference for bilirubin IXα and is abundant in tissues with high heme oxygenase activity. Autophosphorylation of biliverdin reductase causes higher reductase activity, which increases bilirubin production. The consequence is an increased defense against oxidative stress [Stocker, 2004; Vitek & Ostrow, 2009; Vitek & Schwertner, 2007].

The pathway of the heme catabolism can even be noticed in the color change of bruises: while heme shows red or purple color, biliverdin is blue-green and bilirubin yellow [Schmid & McDonagh, 1975].

Mammals, only, degrade biliverdin to bilirubin. In birds, amphibians and reptiles the heme catabolism stops at biliverdin and it is directly excreted. The conversion from the non-toxic biliverdin to the potentially toxic bilirubin, which is additionally expensive in energetic costs remains still an unanswered question. One explanation, though, might be the beneficial role of bilirubin as an antioxidant [Stocker et al., 1987].

2.1.2.2 Transportation and excretion

To provide transportation in the blood circulation, unconjugated bilirubin is strongly bound to albumin. This protein shows high binding affinity and is abundantly available. As a consequence, unbound bilirubin is hardly present

(less than 0.1%). As cytotoxicity is referred to the concentration of unbound bilirubin, it seems, albumin is not only responsible for bilirubin transportation, but also has a detoxification function. Bound to albumin, bilirubin reaches the liver for hepatic uptake. In the hepatocytes bilirubin becomes water-soluble by conjugation with glucuronic acid, glucose or xylose by the enzyme UDP-glucuronosyl-transferase. These mono- and diconjugates are then excreted in the bile. In the intestine, conjugated bilirubin is further hydrolysed by ß-glucuronidases and anaerobic bacteria into urobilinogens and urobilins, which are reabsorbed by the enterohepatic circulation and are excreted by the kidney. Urobilins that remain in the intestine are oxidized to stercobilins and excreted in the feces [Blanckaert, 1981; Stocker, 2004; Vitek & Ostrow, 2009].

2.1.2.3 Pathophysiology

High serum bilirubin concentrations can induce very dangerous conditions, caused by its toxicity. On the other side, some conditions of elevated bilirubin are benign and do not require any clinical treatment.

Normal unconjugated serum bilirubin concentrations range up to 17 µmol/L (1 mg/dL). Concentrations above 30 µmol/L (1.8 mg/dL) are assumed to cause health risks, especially neurological dysfunctions due to toxic bilirubin concentrations in the brain. These high concentrations cause yellow discoloration in sclera and skin and are diagnosed as jaundice (icterus). [Silbernagl & Despopoulos, 2003; Stocker, 2004].

An icterus can be classified as prehepatic, intrahepatic and posthepatic.
The **prehepatic icterus** occurs when hemolysis, and as a consequence bilirubin production, is increased. A defect liver can cause an **intrahepatic icterus**. Drugs, poisons or hepatitis can induce this defect. The **posthepatic icterus** arises after blockage of the bile duct, which can be caused by gall stones or tumors.
Newborns usually develop a hyperbilirubinemia, a physiological jaundice, which is a consequence of the destruction of fetal hemoglobin. If this hemolysis is

increasing, a pathological neonatal jaundice occurs. Untreated, bilirubin concentrations rise and can harm the brain. One kind of treatment is phototherapy, where the newborn is irradiated with light at a wavelength of 400 to 500 nm, making unglucuronated bilirubin water-soluble. Another one is replacement-transfusion [Löffler, 2008; Silbernagl & Despopoulos, 2003].

These forms are referred to as acquired hyperbilirubinemias. Inherited syndromes are Gilbert's, Crigler-Najjar, Dubin-Johnson and Rotor.

Gilbert's syndrome

The Gilbert's syndrome is a relatively frequent benign polymorphism, with mildly elevated serum unconjugated bilirubin concentrations (>17.1 µmol/L). It affects 3 to 17% of the general population, with a greater percentage in men than in women (12.4% and 4.8%). Moreover, mean bilirubin concentrations are significantly higher in males, caused by androgen steroid inhibition of bilirubin enzymatic glucuronidation [Bulmer et al., 2008b; Radu & Atsmon, 2001].
Gilbert's syndrome is a rather harmless condition, which does not cause chronic liver inflammation, histological changes or progressive fibrosis. People with Gilbert's syndrome have a ~30% lower hepatic activity of uridine diphosphate glucuronosyltransferase (UGT1A1) than normally, caused by a mutation in the gene promoter. This polymorphism (UGT1A1*28) shows additional TAs in the TATAA box. However, other factors can lead to increased bilirubin levels such as psychological stress, infection, fasting and physical activity or hemolytic disease [Bosma, 2003; Bulmer et al., 2008b; Danoff et al., 2004; Strassburg, 2010].

Crigler-Najjar syndrome

The Crigler-Najjar syndrome is a more severe form, which can cause death if left untreated. This syndrome is another mutation of the UGT1A gene with (almost) no glucuronidation activity. Two types are described, of which type 1 has no enzyme activity at all and type 2 has an activity below 30% [Strassburg, 2010].

Dubin-Johnson syndrome and Rotor-syndrome

These syndromes are rare but benign abnormalities of the hepatobiliary transport in which direct and indirect bilirubin can be elevated. Both forms require no therapy [Strassburg, 2010].

While highly enhanced bilirubin concentrations can cause severe neurological defects or even death, mildly elevated concentrations (like in Gilbert's syndrome) are assumed to be beneficial, due to the antioxidant properties of unconjugated bilirubin.

2.1.3 Physiologic relevance of bilirubin

The physiologic capacity of bilirubin may be its beneficial effects as an antioxidant. It was shown that bilirubin is a potent antioxidant and can act against free radicals. Free radicals can cause biological damage, referring to as oxidative stress. Oxidative DNA damage is linked to the development of cancer. Consequently, if bilirubin can reduce this damage, it may be protective against cancer.

2.1.3.1 Oxidative stress and reactive oxygen species generation

Oxygen is essential for humans, however, in the oxidative metabolism, O_2 can create toxic substances; namely reactive oxygen species (ROS). ROS are strong oxidants or very reactive free radicals that can be either harmful or beneficial. Their positive effects occur at low or moderate concentrations and include cellular and mitogenic responses. Harmful consequences are biological damages by free radicals, which can lead to oxidative stress. If molecular oxygen takes an additional electron, the superoxide anion radical ($\bullet O_2^-$) is formed. Another reduction forms peroxide anion (O_2^{2-}), which can form hydrogen peroxide (H_2O_2). Adding a third electron arises in the production of the

very dangerous hydroxyl radical (•OH), which is highly reactive [Valko et al., 2007; Kryston et al., 2011; Koolman & Röhm, 2003].

Oxidative stress is an imbalance between oxidants and antioxidants [Sies, 1997]. It is the result of an overproduction of ROS and a deficiency of antioxidants. ROS can harm cell structures, nucleic acids, lipids and proteins, and especially the hydroxyl radical reacts with the DNA and affects DNA synthesis and repair. This oxidative damage, causing harmful influence on genetic material is assumed to be the first step in mutagenesis, carcinogenesis and aging, and is consequently involved in the development of numerous diseases, including cancer, diabetes mellitus, inflammatory diseases and neurodegenerative disorders [Kryston et al., 2011; Valko et al., 2007].
Mutations in DNA can lead to the development of cancer. Elevated DNA damages, arising from oxidative damage, were investigated in cancer. Additionally, in case of a tumor growth, high oxidative stress status was measured [Cooke et al., 2003; Kryston et al., 2011].

2.1.3.2 Antioxidants

Halliwell (2007) defined antioxidants as *"any substance that delays, prevents or removes oxidative damage to a target molecule"*.
Generally, antioxidants should minimize the exposure to O_2 [Halliwell, 2007].
Antioxidants react easily with oxidizing substances and protect cells from oxidation, respectively ROS and free radicals. According to Valko et al. (2006), an antioxidant should scavenge free radicals; chelate redox metals; act synergistically with other antioxidants; activates gene expression; be absorbed easily; have a physiologically appropriate concentration in tissues and body fluids and function in both hydrophilic and/or membrane areas.

There are two kinds of antioxidants: enzymatic and non-enzymatic. Superoxide dismutase, glutathione peroxidase and catalase belong to the enzymatic antioxidants. Vitamin C (ascorbic acid), vitamin E (α-tocopherol), glutathione,

carotenoids, flavonoids as well as bilirubin are non-enzymatic antioxidants [Koolman & Röhm, 2003; Valko et al., 2006].

2.1.3.3 Antioxidant activities of bilirubin

Although traditional chinese medicine is known to have already used bilirubin salts for healing and cure, it was still believed for a long time that bilirubin is either useless or just a toxic compound, especially at high concentrations. In the 1950's though, bilirubin was discovered to have antioxidant properties, by inhibiting oxidation from vitamin A and linoleic acid in the intestinal tract [Rao et al., 2006; Stocker et al., 1987]. Stocker indicated in 1987 that bilirubin might be of physiological importance. Since then, several studies confirmed the antioxidant potential of bilirubin (and biliverdin) [Frei et al., 1988; Neuzil & Stocker, 1994; Stocker & Peterhans, 1989; Vitek & Ostrow, 2009]. It was further reported that bile pigments have anti-complement, anti-inflammatory, anti-viral, anti-apoptotic and anti-mutagenic effects [Bulmer et al., 2008a].

The antioxidant activity of bilirubin arises from its chemical structure. All forms, free, albumin-bound and conjugated bilirubin, have a system of conjugated double bonds and a pair of reactive hydrogen atoms. Especially these reactive atoms make it an antioxidant through H-donation to radicals. This capability results in reduction of oxidation of low density lipoproteins (LDL) and other lipids, which explains its anti-inflammatory properties [Schwertner & Vitek, 2008; Stocker, 2004].

Another aspect is the similar antioxidant activity of bilirubin to ascorbate. While most antioxidants affect only 1e-oxidants, but are poor scavengers for 2e-oxidants, bilirubin and ascorbate are effective against both kinds. Singlet oxygen, hypochlorus acid, quinones and peroxynitrite are examples for 2e-oxidants, scavenged by bilirubin [Stocker, 2004].

As mentioned earlier, heme oxygenase is the enzyme which reduces heme to biliverdin in the heme catabolism. Heme oxygenases are protective enzymes, of

which two isoforms exist: HO-1 and HO-2. HO-1 is derived from the heat-shock protein 32 and induced by oxidative stress, heavy metals, hydrogen peroxide and other factors, while HO-2 is constitutive. Furthermore, HO-1 is important for the antioxidant defense system. The degradation of heme by HO-1 not only results in the antioxidant bilirubin, but also produces iron and carbon monoxide. Iron further generates ferritin, which is beneficial for the endothelium. Carbon monoxide functions as a vasodilator, a second messenger and neurotransmitter in the brain. Several studies reported the positive effects of heme oxygenase, e.g. decreasing ROS production, ischemic liver protection, heart, renal and intestinal ischemia and reperfusion, as well as minimizing atherosclerotic lesions [Jansen et al., 2010; Vitek & Schwertner, 2007]. Besides, heme oxygenase was suggested to have also pro-oxidant effects [Ryter & Tyrrell, 2000].

2.1.3.4 The theory of a redox cycle of bilirubin and biliverdin

Although the antioxidant properties of bilirubin seem clear, it is yet not completely solved *how* it protects cells from oxidation - apart from structural aspects. Lately, the theory of a redox cycle of bilirubin and biliverdin was discussed. In this cycle, bilirubin is oxidized to biliverdin and then reduced back to bilirubin by the ubiquitous biliverdin reductase. This assumption was based on a report by Doré et al. from 1999, which showed that even nanomolar concentrations of bilirubin protected cells from H_2O_2 toxicity *in vitro*. The cycle-theory suggested that bilirubin molecules act again and again and therefore could explain high effectiveness even at low concentrations.

The redox cycle was investigated by Maghzal et al. (2009) under various conditions and these results could not show that bilirubin generates biliverdin, nor that biliverdin reductase is an important protector of cells. Yet, there was suspect of an anti-apoptotic bilirubin function. McDonagh (2010) studied the redox cycle as well, but couldn't find proof to it either. Moreover, McDonagh termed this theory as highly doubtful. Data misinterpretation and impure bilirubin may have caused a false assumption.

2.1.3.5 Measurements *in vitro*

Antioxidant properties were examined by Stocker et al. (1987) in a lipid *in vitro* system. Linoleic acid was oxidized by a free radical chain and formed linoleic acid hydroperoxide (LOOH), which can be measured. Bilirubin and biliverdin at very low concentrations reduced the formation of LOOH significantly. Furthermore, bilirubin inhibited partially the chain reaction. These results suggest that bilirubin can scavenge peroxyl radicals. For physiological relevance, bilirubin was then compared with ß-carotene and α-tocopherol. Bilirubin showed similar rates of antioxidant activity and at low oxygen concentrations bilirubin could even beat α-tocopherol.
As already mentioned above, most circulating bilirubin in humans is bound to albumin and results in increased specificity and reactivity. Albumin-bound bilirubin inhibits oxidative damage of fatty acids, which are transported on albumin. Moreover, it protects proteins from ROS caused damage and at normal concentrations albumin-bound bilirubin is an efficient radical scavenger in humans that protects lipids as well as proteins [Stocker, 2004].

Two studies showed *in vitro* evidence for an anti-cancer effect of bilirubin. Keshavan et al. (2004) showed that bilirubin had a reducing effect on the viability of colon adenocarcinoma cell lines at physiologic concentrations (0-50 µM) and also stimulated apoptosis in HCT15 colon adenocarcinoma cells. These observations were only seen with the unconjugated form of bilirubin. Rao et al. (2006) investigated the effect of bilirubin on human carcinoma cell lines. Cells were prepared, treated with physiological bilirubin concentrations (10 µg/mL) and incubated. In hepato-carcinoma cells an anti-oxidative effect was noticed and, surprisingly, in TMK-1 cell lines a pro-oxidant effect. Other antioxidants had no effect on TMK-1 cell lines. These results lead to the assumption that bilirubin has an anti-cancer activity.

Bulmer et al. (2007) studied on the anti-mutagenic properties of bile pigments. Unconjugated bilirubin, bilirubin ditaurate and unconjugated biliverdin were tested using the Ames Salmonella test. Bilirubin as well as biliverdin showed

anti-mutagenic activity and were able to inhibit genotoxic effects of several mutagens. Moreover, antioxidative activity was tested and found positive in bilirubin and biliverdin.

While the antioxidant activity of bilirubin seems to be proven in *in vitro* systems, its properties are not so clear *in vivo*. Although assumed to be antioxidative *in vivo* as well, there is only indirect evidence. To get more information, the effect of altered pigment concentrations on established *in vivo* markers, e.g. oxidized lipids, should be investigated [Stocker, 2004]. One report that indicates antioxidant bilirubin function *in vivo* though, was published by Dennery et al. (1995). They investigated oxidative stress in Gunn rats and nonjaundiced control rats after exposure to oxygen. Thiobarbituric acid and lipid hydroperoxides were reduced in Gunn rats and therefore support the antioxidative theory.

2.1.4 Epidemiological studies

Antioxidant activity and beneficial effects of bilirubin have been in focus of researchers for many years. In the following section epidemiological studies showing evidence to these properties are presented.

2.1.4.1 Cardiovascular disease

One of the first reports that showed an association of bilirubin and coronary artery disease (CAD) was published in 1994 by Schwertner et al. 619 male American pilots and navigators were examined and an inverse relation between low total serum bilirubin and an increased risk of CAD was found. This relation was statistically significant and as strong as the association with smoking or systolic blood pressure. Although bilirubin was assumed to be an antioxidant, yet it was not known that bilirubin could prevent or protect from CAD.

This inverse relationship between total bilirubin and CAD prevalence was shown again recently. In a study, including 100 healthy and 100 subjects

diagnosed with CAD, it was reported that bilirubin was decreased in CAD patients compared to controls. In other words, subjects with low serum bilirubin concentrations had a higher risk for CAD [Ghem et al., 2010].

A connection between serum bilirubin and cardiovascular disease (CVD) was found in an association study of the Framingham Heart Study. The Framingham Heart Study started in 1948, investigating on the risk factors of CVD in the general population. Among these, 1780 subjects with an average age of 36 years were analysed retrospectively, as these subjects had given genotype information for UGT1A1 polymorphism. A significantly reduced risk for CVD and coronary heart disease was noticed in subjects with UGT1A1*28, which causes mildly elevated serum bilirubin levels [Lin et al., 2006].

Another study investigated effects of bilirubin on individuals with Gilbert's syndrome. 50 people with this hyperbilirubinemia were compared to a control group and the prevalence for ischemic heart disease was 2% in relation to 12.1%. It was suggested that unconjugated bilirubin may prevent ischemic heart disease development [Vitek et al., 2002].

In a study by Bulmer et al. (2008b) nine individuals with Gilbert's syndrome were compared to 12 controls. The aim was to find mechanisms that are responsible for the protection from CVD at high bilirubin concentrations. Gilbert's syndrome was identified by circulating unconjugated bilirubin levels >17.1 µmol/L (1 mg/dL). However, this study found no differences within the groups in their antioxidant status and oxidative stress, but a significantly reduced susceptibility of serum to oxidation was noticed in Gilbert's syndrome patients.

Yesilova et al. (2008) investigated if high serum bilirubin concentrations can protect LDL from oxidation. Therefore, 17 patients diagnosed with Gilbert's syndrome and 15 matched, healthy controls were compared. There were no significant differences between the groups. However, LDL oxidation

susceptibility experiments showed a longer lag phase in Gilbert's syndrome patients compared to controls. Also, serum bilirubin concentrations and LDL oxidation susceptibility values were significantly correlated. These findings might indicate that elevated bilirubin levels are related with low LDL oxidation, and this is suggested to protect from coronary heart disease.

In a study by Tapan et al. (2011) 42 male persons with Gilbert's syndrome were compared to 52 matched healthy controls. Small dense LDL cholesterol, oxidized LDL and high-sensitivity C-reactive protein levels were examined and all three values were lower in the Gilbert's syndrome group than in controls. Oxidized LDL is involved in the development of atherosclerosis, consequently it was assumed that elevated bilirubin might decrease the risk of this condition.

2.1.4.2 Cancer

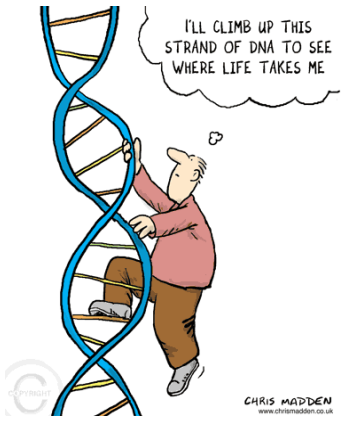
In the population from the Belgium Inter-university Research on Nutrition and Health (BIRNH) study, Temme et al. (2001) investigated on 5460 men and 4843 women, aged between 25 and 74 years, the association between serum bilirubin and cardiovascular and cancer mortality. Originally, the BIRNH study investigated the relationship between diet and mortality and was performed from 1981 to 1984. Ten years after the first data collection, vital status and cause of death of participants were ascertained. Average serum bilirubin concentrations in men were 0.44 mg/dl (range: 0.1–3.8 mg/dl) and 0.35 mg/dl (range: 0.1–2.8 mg/dl) in women. Results showed that men with higher serum bilirubin concentrations had a 58% lower risk of cancer mortality. Women showed same trends (24% lower risk), but no statistic significance.

A similar evaluation was done by Zucker et al. (2004) in the United States of America. The Third National Health and Nutrition Examination Survey (NHANES III) is a huge database performed between 1988 and 1994 including 40 000 people. Among these, 16 865 subjects had given serum bilirubin data, with significantly lower concentrations in women (0.52 ± 0.003 mg/dL) compared to men (0.72 ± 0.004 mg/dL). A significant inverse relationship

between serum bilirubin concentrations and colorectal cancer was investigated. An increase of each 1 mg/dL total bilirubin caused a decrease in cancer risk. As the NHANES III database did not differ between conjugated and unconjugated bilirubin it can not be sure that the effect originates from the unconjugated form, however, the study authors suspected so. These investigations indicate that bilirubin protects from cancer.

Horsfall et al. (2011) published a report comparable to the above ones recently. Information was gained from The Health Improvement Network (THIN) database from the United Kingdom, including more than 7 million people. 504 206 individuals were included with a median follow-up of 8 years. Average serum bilirubin concentrations in men were 0.64 mg/dl (interquartile range: 0.47–0.88 mg/dl) and 0.53 mg/dl (interquartile range: 0.41–0.70 mg/dl) in women. It was shown that higher concentrations of bilirubin decreased the risk of lung cancer and all-cause mortality. These findings were similar both in men and women.

2.2 Oxidative DNA damage



2.2.1 DNA & DNA damage

2.2.1.1 DNA

Deoxyribonucleic acid (DNA) is a polymer molecule, made of nucleotides. DNA contains and storages the genetic information of all living cells. Therefore, changes (mutations) in the DNA sequence can lead to damage in the molecule. While on one side mutations are important for biological evolution, on the other side too much can be harmful. Mutations are caused by physical and chemical influences or by random errors of DNA replication or recombination [Koolman & Röhm, 2003].

2.2.1.2 Mitosis and cell cycle

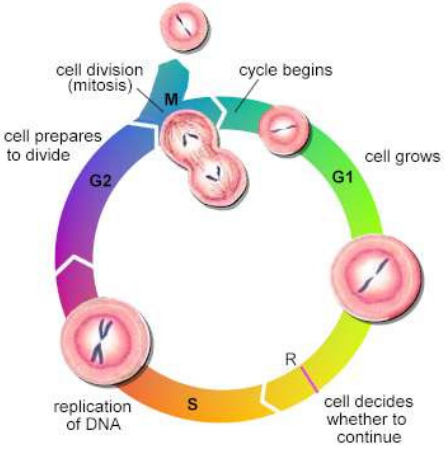


Figure 3. The cell cycle [David Darling, 2011].

Every eukaryotic cell has to go through mitosis and the cell cycle. Mitosis is the process of cell division. The cell cycle can be divided into four phases: G_1, S, G_2 and M. The G_0 phase is a resting phase, in which cells can endure for a long time. After the last mitosis, proliferating cells enter the G_1 phase, where the cell

is growing and synthesizing its materials. The next phase is the S phase, which is entered by the influence of growing factors. In this phase, DNA replication takes place and in the end the cell is tetraploid. After that, cells enter the G2 phase, which is relatively short. Finally, cells enter the M phase. Mitosis has four stages: prophase, metaphase, anaphase and telophase. The division of the cytoplasm, resulting in two daughter cells with genetic identical material is termed cytokinesis. At prophase the nuclear envelope disintegrates, the nucleolus disappears and the centrioles, when present, duplicate and migrate to opposite poles of the cell. At metaphase the chromosomes line up in the middle of the cell, called the metaphase plate. This alignment of the chromosomes marks the end of the metaphase. At anaphase chromatids separate and are pulled to opposite poles of the cell. At telophase the chromosomes uncoil and begin to carry out their physiological functions. A nuclear envelope re-forms about each set of chromosomes, nucleoli form and cytokinesis takes place. Now the cells enter the G1 phase of the cell cycle and the process can start again.

The cycle needs to be strictly controlled. If cells enter a phase without completing the previous one, cell death is likely. Cyclins and cyclin-dependent kinases (CDKs) control the cell cycle phases. To prevent that DNA damaged cells enter the cycle the tumor suppressor proteins p53 and retinoblastom protein work together with cyclin-dependent kinases [Löffler, 2008; Tamarin, 1993].

2.2.1.3 DNA damage

DNA damage arises from several mutagens, free radicals and reactive oxygen. There are different kinds of DNA damage and in the best case they get repaired or eliminated.

DNA damage depends on the type of mutagen and on where it reacts. Mutagens can react with DNA directly (e.g. ionizing radiation, oxidizing chemicals, UVA solar light) or indirectly (e.g. hydroxyl radical) via protein binding and formation of free radicals. Free radicals are produced when a cellular imbalance occurs, and oxygen is available. This can result in reactive

oxygen species. Different types of DNA damage can occur as single-strand breaks and double-strand breaks, DNA adducts or gene-, chromosome- and genome mutations [Mateuca et al., 2006; Kryston et al., 2011].

2.2.2 Measuring DNA damage

Although there are mechanisms to prevent DNA damage, it still can occur. These damages can be detected with specific methods, e.g. the chromosome aberration assay and the micronucleus assay. Today, the micronucleus assay is frequently used and can detect different kinds of DNA damages. These damages are measured in lymphocytes and occur as micronuclei, nucleoplasmic bridges and nuclear buds.

2.2.2.1 The chromosome aberration assay

The chromosome aberration assay in peripheral blood lymphocytes (PBL) has been used in the past 30 years to measure genotoxic carcinogens. Several studies showed an association between chromosomal aberrations and an increased risk of cancer. However, this method is in disadvantage compared to the nowadays more frequently used micronucleus assay [Mateuca et al., 2006; Bonassi et al., 2008].

Chromosomal aberrations are either changes in the chromosome structure or in the chromosome number.

Structural changes are generated by DNA breakage, replication, synthesis inhibition and other mechanisms. They can be differed in chromosome-type aberrations and chromatid-type aberrations. Chromosome-type aberrations occur from incompletely repaired or unrepaired double strand breaks and chromatid-type aberrations from base modifications or single strand breaks. These aberrations are then repaired, incorrect repaired or not repaired at all. On microscope slides the incorrect repair as well as the not repaired damages can be observed.

Numerical changes represent modifications in the normal chromosome number, termed aneuploidy or polyploidy [Mateuca et al., 2006].

2.2.2.2 The cytokinesis-block micronucleus assay

The cytokinesis-block micronucleus assay (CBMN) was developed to measure micronuclei. In the past years, scoring of nucleoplasmic bridges and nuclear buds was established and became more important. Hence, the CBMN assay has turned into a "cytome" concept in which every cell is scored. Besides MN, NPB and NBUD, apoptosis and necrosis as well as mono- bi- and multinucleated cells are counted. All these formations give information about chromosome breakage, chromosome loss, non-disjunction, cell death and cytostasis in human lymphocytes. The cytokinesis inhibitor cytochalasin-B, added 44 hours after the cultivation start, blocks cell division at the stage of binucleated cells, to which scoring is restricted.

Due to its statistical power and relative simpleness, the CBMN assay has become a standard method for testing genetic toxicology and measuring chromosomal DNA damage in humans. Moreover, it has been proposed, that the frequency of MN can estimate the risk of cancer and cardiovascular disease [Fenech & Bonassi, 2011; Fenech, 2006; Mateuca et al., 2006].

Micronuclei (MN) are biomarkers for chromosome breakage (acentric fragments) and/or chromosome loss. They occur in once-divided binucleated cells and have the same morphology as the main nucleus, by the exception that they are smaller.

MN from acentric chromosome or chromatid fragments can result from several mechanisms. Misrepair of DNA double-strand breaks can produce chromatid and chromosome fragments. A few fragments originate from unrepaired double-stranded DNA breaks. Another mechanism causing MN formation is simultaneous excision repair of damaged or wrong bases incorporated into the DNA. Especially uncompleted gap-filling can cause DNA double-strand breaks and consequently MN. Additionally, MN can arise from leftover chromosome material from nucleoplasmic bridges (see later).

MN can originate from whole chromosome malsegregation at anaphase. This is caused by chromosomes that were not included in the daughter nuclei during mitosis and could therefore not reach the spindle poles. One mechanism is hypomethylation of cytosine in centromeric and pericentromeric repeat sequences. Another origin might be defects in kinetochore proteins, which are required for the meshing of chromosomes with the spindle. Defects in mitotic spindle assembly, mitosis check point defects and abnormal centrosome amplifications are mentioned as well. More recently discussed are dicentric chromosomes resulting from telomere end fusions [Fenech, 2000; Fenech et al., 2011].

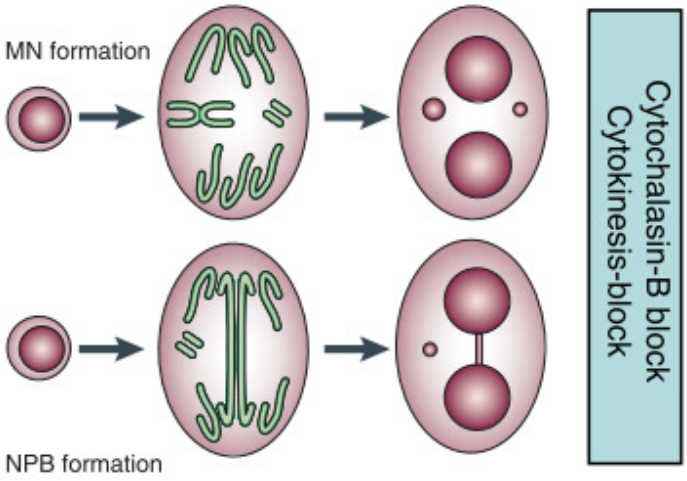


Figure 4. MN and NPB formation in cells undergoing nuclear division. MN originate from either lagging whole chromosomes or acentric chromosome fragments. NBP originate from dicentric chromosomes that may be caused by misrepair of double strand DNA breaks or telomere end fusions. These events can only be observed in cells completing nuclear division, which are recognized by their binucleated appearance after cytokinesis blocking with cytochalasin-B [Fenech, 2007].

Nucleoplasmic bridges (NPB) are a biomarker of chromosome rearrangement and arise when centromeres of dicentric chromosomes are pulled to opposite poles of the cell during anaphase. These dicentric chromosomes develop from misrepair of chromosome breaks and can result in a NPB. They can also originate when the telomere ends are merged together, caused by adverse arrangement of telosome protein structure. Generally, NPB would break during

cytokinesis, however, in the micronucleus assay they are still visible, since the cytokinesis inhibitor cytochalasin-B is used. This poison stops cell division at the binucleated stadium, where NPB are stable.
NPB frequency is growing by exposure to endogenous oxidants, ionising radiation, polycyclic aromatic hydrocarbons, cigarette smoke carcinogen, vanadium pentoxide and deficiencies in folate and selenium [Fenech et al., 2011].

Nuclear buds (NBUD) are a biomarker of gene amplification. They occur during the S phase of the cell cycle (nuclear budding) and have the same morphology as MN, indeed they are still connected to the nucleus. Similar to MN and NPB, NBUD formation show different ways of origin. They are predominantly built from interstitial or terminal acentric fragments. Another possibility is that NBUD are leftovers from DNA after nuclear division or they could also be formed by eliminating unnecessary chromosomes. Potentially, NBUD on one or both nuclei can arise after a NPB is broken [Fenech et al., 2011; Mateuca et al., 2006].

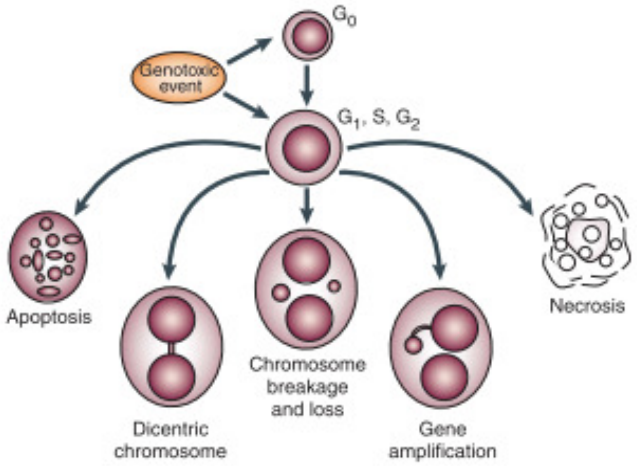


Figure 5. The various possible fates of cultured cytokinesis-blocked cells following exposure to cytotoxic/genotoxic agents. Using these biomarkers within the CBMN assay, it is possible to measure the frequency of chromosome breakage (MN), chromosome loss (MN), chromosome rearrangement, for example, dicentric chromosomes (NPB), gene amplification (NBUD), necrosis and apoptosis. In addition, cytostatic effects are readily estimated from the ratio of mono-, bi- and multinucleated cells [Fenech, 2007].

Apoptosis is a genetically programmed cell death leading to degradation. Apoptosis can be identified by its morphology including change of cell membrane, shrinking of the nucleus, chromatin condensation and chromosomal DNA fragmentation. Macrophages and other cells recognize apoptotic cells and remove them through phagocytosis, without causing inflammation [Koolman & Röhm, 2003].

In contrast, **necrosis** is caused by physical or chemical damages. Cells can swell and burst and this can lead to an inflammation. While apoptosis can be considered to be beneficial, necrosis refers usually to “unnatural” cell death [Koolman & Röhm, 2003].

2.2.3 Influencing factors for DNA damage markers

The influences of age and gender on MN in PBL were already discovered in the mid-1980s and steadily approved in numerous studies until today. Nutrition also has a huge influence on MN formation. So far, nine micronutrients were identified to have an effect on genome stability in humans. Some studies showed a reduction of MN with moderate exercise, however, the effects of extreme exercise are still unclear. The relevance of smoking is yet unexplained, as some studies reported increased MN frequencies in smokers and others don’t. However, heavy smokers do have significantly elevated MN frequencies. High alcohol consumption is considered to have an increasing effect on MN formation as well [Fenech & Bonassi, 2011].

2.2.3.1 Age and gender

MN frequencies increase with age in both males and females but generally females have higher values.

In 1999, Peace and Succop analyzed 11 studies from different countries. All, except one of them, used the CBMN assay. 10 studies observed a positive correlation between age and MN frequency. Regarding the gender effect, 3 out

of 11 studies did not provide data, 5 proposed no differences and 3 showed differences with MN being greater in females.
In 2001, Bonassi et al. evaluated data from 25 laboratories from 16 countries, including almost 7000 subjects (HUMN-project). They confirmed that MN increased with age in both genders. Women showed a 19% higher frequency in MN than men.
In 2007, Wojda et al. published that both men and women had higher MN frequencies as they got older and higher rates were found in females.
Similar observations can be found in several other publications [Coskun et al., 2011; Costa et al., 2007; Garaj-Vrhovac et al., 2008; Mateuca et al., 2006].

Many factors can cause the age-increasing effect of MN. There are cumulative effects of gene mutations that are implied in DNA repair, chromosomal division and cell cycle checkpoints, or alterations of chromosomes due to the exposure to genotoxins, poor nutrition, stress, sickness and other factors. Generally, an unhealthy lifestyle is considered to cause higher MN formation. The reason for more MN in women might be explained by the fact that females have two X chromosomes (while men have one) and these tend to get lost as MN easier than other chromosomes. 72% of the observed MN in women can be accounted by the X chromosome [Fenech & Bonassi, 2011; Fenech et al., 2011].

2.2.3.2 Folic acid and vitamin B12

Folic acid and vitamin B12 are important factors in the DNA metabolism. Folic acid is relevant for the synthesis of dTMP from dUMP. If there is a deficiency of this micronutrient, uracil is incorporated into DNA instead of thymine. This wrong incorporation can cause point mutation, single- and double-strand DNA breaks, chromosome breakage and MN. Folic acid and vitamin B12 are important for the synthesis of methionine and S-adenosyl methionine (SAM). SAM determines gene expression and DNA conformation. Vitamin B12 deficiency causes reduced SAM synthesis, leading to uracil incorporation into DNA.

In summary, a deficiency of folic acid and vitamin B12 can lead to greater DNA damage and altered methylation of DNA. These are both risk factors for cancer. Also, deficiencies raise levels of homocysteine which is a risk factor for cardiovascular disease [FENECH, 2001].

In 2005, Fenech et al. examined in 190 healthy subjects the relationship between diet and genome damage. Five micronutrients were identified (retinol, vitamin E, folate, nicotinic acid and calcium) to be protective and reduce MN formation. Concerning folate, its importance for chromosomal stability could be shown and that folate deficiency caused elevated MN frequencies.
A study from Stopper et al. (2008) investigated the effects of folate acid and vitamin B12 supplementation on 27 dialysis patients. They measured MN frequencies each three times before and after supplementation and found significant reductions of genome damage after the supplementation.
Minozzo et al. (2010) investigated genotoxic damage in lead-exposed workers, considering folate and vitamin B12. Lead-exposed subjects showed significantly higher MN frequencies than the control group. The study was conducted in Brazil, where flour is enriched with folic acid since 2004. Therefore, B12 and folic acid concentrations were similar in both groups. Subjects with equal or higher folic acid levels than mean showed significantly more MN and NPB. Previous studies showed the same effect, suggesting that folate concentrations increase with age. Referring to the authors, these results might suggest that only high folate concentrations are not enough for reducing MN formation.
Milic et al. (2010) examined folate and vitamin B12 concentrations in 36 male, healthy subjects for DNA damage. These findings showed that individuals with lowest vitamin B12 concentrations had highest MN frequencies. This leads to the conclusion that vitamin B12 is involved in DNA integrity. These results may also indicate that the positive effect of folic acid and vitamin B12 is measureable in undernourished persons as well as in healthy ones.

2.2.4 DNA damage markers as predictor of cancer risk

Back in 1902, Theodor Boveri made a remarkable finding, when he linked chromosomal abnormalities with cancer. Mutation and selection are principally important evolutionary mechanisms, however, they can cause chromosomal aberrations and consequently lead to cancer development. Most tumors show a huge amount of chromosomal aberrations which reflect an accumulation of genetic damage. Consequently, genomic instability indicates the process of carcinogenesis. The intensity of damages can be measured and reflect the cancer risk. MN, NPB and NBUD are caused by chromosomal rearrangements, altered gene expressions or aneuploidy – effects of chromosomal instability which are often seen in cancer. Therefore, these biomarkers can predict the risk of cancer, which was already shown in several studies. There is evidence that cancer patients have higher frequencies of MN, NPB and NBUD [Bonassi et al., 2011].

2.2.4.1 Micronuclei in healthy individuals and cancer patients

A first association of chromosomal aberrations and cancer risk was made by a Nordic study group. They measured chromosomal aberrations, sister chromatid exchange or MN in PBL in 3182 subjects between 1970 and 1988. In a follow-up period lasting until 1991 they observed 85 cancer incidents. They found a significant positive trend with chromosomal aberrations and cancer risk. No relation was found in MN frequencies and cancer risk, due to the lack of data, as only 11 cancer cases were examined for MN formation before their diagnosis [Hagmar et al., 1994].

In a huge analysis of 6718 subjects from 10 countries it was indicated that MN in PBL were a predictive biomarker of cancer risk even in a healthy population. Bonassi et al. (2007) compared data from 20 laboratories published between 1980 and 2002, by splitting them into groups of low, medium and high MN frequencies. In a follow-up period of eight years, carcinogenesis and mortality of subjects were investigated. Significant increases of cancer incidences were found in the medium and high group, independent from nationality. Hence, both

groups showed a decreased cancer-free survival, compared to the low group. Although an association of MN and cancer was observed with different types of cancer, only urogenital and gastro-intestinal cancers were statistically significant [Bonassi et al., 2007].
In a similar study from Italy, published in 2008, it was aimed to analyze cancer in healthy individuals. Between 1991 and 1993 blood samples from 1650 subjects were collected. A follow-up period until January 2005 was performed. The findings showed that 111 people died, of which 52 persons had cancer. Their samples (only 49 could be analyzed) were then compared to 101 matched controls. Indeed, cancer cases had significantly higher MN frequencies than controls, which indicates that MN can predict cancer [Murgia et al., 2008].

In a meta-analysis, results from 25 studies were evaluated for MN frequencies in untreated cancer patients. Compared to cancer-free controls, they had a 28 to 64% higher rate of MN [Iarmarcovai et al., 2008].

In a study published in 2010, samples from 44 untreated cancer patients were compared to 40 disease-free controls. Mean MN frequencies were significantly higher in patients than in controls, independently from cancer sites [Milosevic-Djordjevic et al., 2010].

In a recently published study, 346 patients with pancreatic cancer were compared to 449 healthy persons. MN frequencies of cancer patients were significantly higher than in controls, with a higher cancer risk in men than in women [Chang et al., 2011].

In another report, 59 patients with diagnosed head and neck cancer were compared to 34 healthy first-degree relatives (including parents, siblings or sons/daughters). Data were also collected from 31 healthy volunteers who did not have any cancer history in their families, and compared with cancer cases. Highest values for MN frequencies were found in cancer patients and were significantly higher than in relatives and controls [Burgaz et al., 2011].

The findings from the presented studies give evidence that elevated frequencies of MN can be associated with an increased cancer risk and that the CBMN assay is a reliable tool for the cancer risk assessment.

2.2.4.2 Bilirubin and DNA damage

So far, only one study has analyzed micronuclei frequencies and compared them to elevated serum bilirubin levels. It should be noted that bilirubin concentrations were not measured, but indicated by UGT1A1 polymorphism.

In this study it was hypothesized that UGT1A1 polymorphism, causing elevated bilirubin concentrations, can protect from oxidative damage. Individuals, who are homozygous for 7- and 8-TA repeat allele have a mild hyperbilirubinemia (Gilbert's syndrome), while repeats of 5- and 6-TA does not cause elevated bilirubin concentrations. 101 healthy persons, aged between 19 and 53 years, were recruited and analyzed for UGT1A1 polymorphism. They measured kinetochore-positive and -negative MN frequencies in 63 subjects (48 smokers and 15 non-smokers) and found a trend indicating that individuals with lower bilirubin have higher kinetochore-positive MN frequencies, but not significantly [Grant et al., 2004].

Smoking can lower serum bilirubin concentrations and bilirubin concentrations were not directly measured, therefore, it is not possible to compare these results with the present study. Also, Grant et al. used a slightly different method for the MN measurement than at the present study.

3 MATERIALS and METHODS

3.1 Study Design

The aim of the present study was to establish whether the antigenotoxic effects of bilirubin prevent micronuclei formation in individuals with moderately elevated circulating serum bilirubin concentrations (Gilbert's syndrome). This study had a cross-sectional design and was in total performed over a period of two years.

There is little information on oxidative DNA damage in people with Gilbert's syndrome (GS), therefore this study aimed to investigate levels of DNA stability in GS and matched control subjects (i.e. people with normal bilirubin concentrations). Furthermore, it was hypothesized that lymphocytes from GS subjects would be protected from free radical induced DNA damage due to elevated BR levels. The results were expected to show whether moderately increased unconjugated serum bilirubin levels have beneficial effects on biomarkers of oxidative damage. Additionally, oxidative stress, inflammation and cardiovascular disease related parameters as well as the nutritional status were measured. The links between these factors and DNA damage were also investigated.

For the screening, blood was taken from the subjects after the patients agreement was filled. A questionnaire was administered, to check whether the subjects met the inclusion or exclusion criteria (see criteria below). When the subjects met the inclusion criteria, they were invited to give a second sample at the General Hospital Vienna (AKH). The participants were randomized in GS and controls.

Participants were advised to fast and only ingest 400 kcal (between 9 am and 5 pm, followed by an overnight fast), before the second, main blood collection. It is well known that as a consequence of fasting, serum bilirubin levels rise, especially in people with GS [Owens et al., 1973]. The next morning, blood, urine samples and buccal cells were collected. The blood samples were taken by a trained clinician at the AKH and were then transported to the Department

of Nutritional Sciences. Samples were continuously protected from light to prevent bilirubin oxidation.

A coding system (e.g. GS_01 for first participant) was used to de-identify subjects and assisted in removing bias from the analysis. Researchers did not know whether subjects had high or low serum bilirubin concentrations.

Study population

This study was approved by the Human Ethics Research Committee of the AKH (Ref. No.: 274 / 2010). A patient agreement was issued to participants and they had to declare their approval. All samples were collected from April to September 2010.

A total of 100 subjects, aged between 20 and 80 years, were recruited for the study. Participants were identified as GS, based on their serum unconjugated bilirubin concentrations >17.1 µmol/L (>1 mg/dL) determined by high-performance liquid chromatography (HPLC). For the clinical definition of GS, normal serum liver enzyme activities and blood counts were required in addition to enhanced bilirubin levels. 45 of these participants were diagnosed with GS and 39 participants were then allocated into the control group. 16 subjects could not be assigned to one of the groups, due to their unclear bilirubin levels and were just included for statistical analysis using tertiles of bilirubin serum concentration.

Subjects were recruited partly by doctors in the AKH and by advertisements posted in universities, pharmacies or hospitals, as well as on internet platforms and in newspapers. For their participation, subjects were refunded with € 50,-.

Inclusion criteria

- 20 - 80 years
- total serum bilirubin >20.52 µmol/L (>1.2 mg/dL) in GS subjects
- unconjugated serum bilirubin >17.1 µmol/L (>1 mg/dL) in GS subjects
- total serum bilirubin <20.52 µmol/L (<1.2 mg/dL) in controls
- unconjugated serum bilirubin <17.1 µmol/L (<1 mg/dL) in controls
- γ-glutamyltranspeptidase in blood <100 IU

- alanin-aminotransferase in blood <100 IU
- aspartat-aminotransferase in blood <100 IU
- moderate physical activity

Exclusion criteria

- younger than 20 years or older than 80 years
- cardiovascular diseases
- hepatitis B/C
- any other liver diseases
- cholelithiasis
- hemolysis
- chronic kidney diseases
- past or present cancer
- diabetes mellitus
- supplementation with antioxidants in the past four weeks before the first blood sample
- medication that influences liver values (e.g. Probenecid, Rifampicin)
- people with organ transplants
- current smoking (>5 cigarettes per day)
- alcohol consumption (>7 drinks per week)
- competitive athletes (>10 hours training per week)

3.2 HPLC analysis

Bilirubin is sensitive to light and oxygen [Zelenka et al., 2008] therefore, all preparations had to be done fast and ideally under light protection. Whenever possible, dark tubes were used, closed immediately and serum was kept at 4 °C.

Bilirubin is known to have three isomers: IIIα, IXα, and XIIIα. The HPLC chromatogram shows one major peak, known as the IXα-isomer, and two smaller ones. According to Zelenka et al., in commercial bilirubin the IXα-isomer takes about 92% of the peak area, while bilirubin IIIα and bilirubin XIIIα have only 4 % each. This is as well valid for the analysis within this study and can be seen in Figure 6, which shows a chromatogram of a standard solution. By contrast, in human serum only IXα-bilirubin is detectable, as shown in Figure 7.

The following method was modified from the HPLC protocol of Bulmer et al. (2008b).

The serum unconjugated bilirubin concentration was quantified using HPLC and a photo diode array detector. The serum was separated using a C18 reverse phased column at a flow of 1 mL/min. From each sample and standards 50 µL were injected into HPLC column for separation. A retention time of approximately 12 minutes was obtained. Some of the samples, randomly chosen, were analyzed in duplicates. The evaluation was done via standard curve.

3.2.1 HPLC, reagents and equipment

HPLC adjustments, reagents and equipment are summarized in Table 1, Table 2 and Table 3.

Table 1. HPLC adjustments.

High-Performance Liquid Chromatography	
Pump	LaChrom Elite, Hitachi L-2130
Sampler	LaChrom Elite, Hitachi L-2200, Auto-Sampler 4 °C, Injection Volume: 50 µL
Oven	LaChrom Elite, Hitachi L-2300 Column Oven, Temperature: 35 °C
Detector	SPD-M20A, Shimadzu, Photo diode array detector
Wavelength	450 nm. Cell Temperature: 35 °C
Column	Fortis F18-050703 HPLC column, 4.6 x 150 mm, 3 µm
Flow Rate	1.0 ml/min
Pressure	160 bar (in average)
Software	EZ Chrome, Hitachi

Table 2. Reagents for HPLC analysis.

Name	***Manufacturer***	***Factory number***
DMSO	Sigma Aldrich	D 4540
Glacial acetic acid	Sigma Aldrich	A 9967-500G
HPLC grade methanol (purity 99.9%)	Sigma Aldrich	34860
HPLC grade water	Sigma Aldrich	95304
Bilirubin (alpha)	Frontier Scientific	B 584-9
N-dioctylamine	Sigma Aldrich	D 20 1146-100G

Table 3. Equipment for HPLC analysis.

Name	***Manufacturer***
Blood collection tubes Vacuette®	Greiner bio-one
Centrifuge	Thermo Scientific, Megafuge 40
Centrifuge	Eppendorf 5413R
Microcentrifuge tubes, dark, 1.5 mL	Eppendorf
Ultrasound-bath	Bandelin Sonorex RK 100

3.2.2 Preparation of mobile phase and standard solutions

Mobile Phase

The mobile phase for the HPLC-analysis was prepared as follows.

150 mL of methanol were poured in a conical flask, placed on a scale and zeroed. As methanol evaporates, the next step had to be quick: 24.2 g dioctylamine were added to the methanol. For smaller amounts, a glass pipette was used. The dioctylamine starts to precipitate when it comes in contact with air. To minimize this reaction, the lid had to be put on as soon as finished. To avoid evaporation of methanol, the flask was covered with a parafilm, when finished with this step. Then, a small beaker was placed on the scale in the fume cupboard and zeroed. Using a glass pipette, 6.01 g of glacial acetic acid was added step by step to the methanol/dioctylamine-mixture. The reaction is exothermic which heats up the flask slightly. 800 mL of methanol were poured in a 1 L flask and the methanol/dioctylamine/acetic acid-mixture was added. Finally, 50 mL of HPLC grade water was added and all components were mixed together.

Column activation

The column was flushed by pure methanol about three to four hours, and then gradually mobile phase was added over a period of 15 to 20 minutes before 100% mobile phase was reached. The column should run 100% mobile phase for three to four hours, before injecting the first sample.

Preparation of standard solutions

Exactly 5.85 mg of commercial bilirubin were weighed out on a small weigh boat. With a pipette bilirubin was transferred by DMSO into a 20 mL volumetric flask and exactly filled up to the mark. By gentle shaking and using the ultrasound bath all constituents were dissolved. The resulting concentration was 500 µM and was equivalent to the standard solution one (S1).

For all other dilutions the scheme, shown in Table 4, was followed and flasks were exactly filled up to the mark with DMSO. Bilirubin has its best solubility in

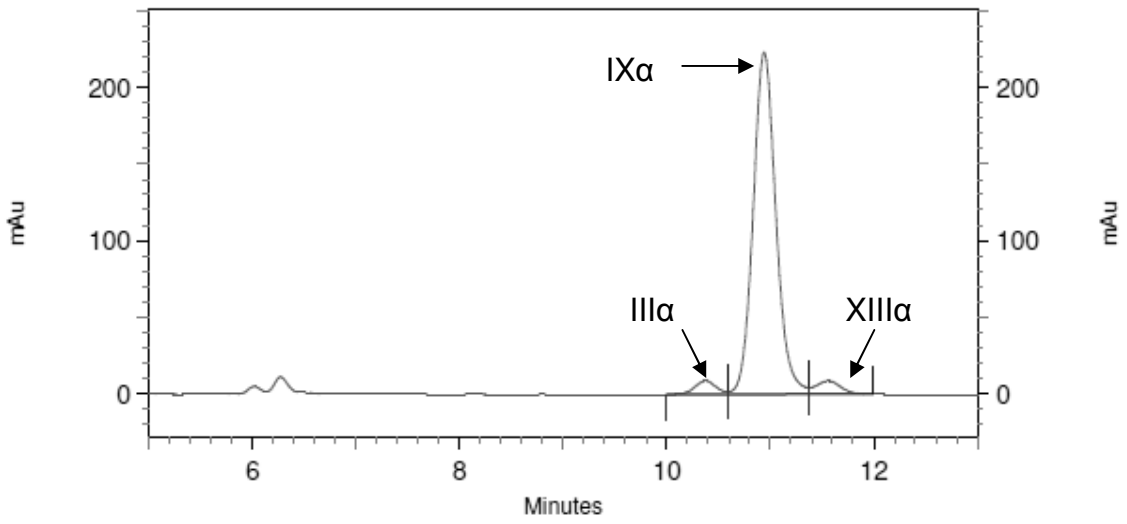
DMSO, is good soluble in chloroform and difficult in water, therefore, DMSO was used in the trial.

Table 4. Scheme for standard solutions.

Standard solution		***[V] mL***	***[c] µmol/l***
S1	5.85 mg bilirubin	20	500
S2	2 mL S1	5	200
S3	500 µL S2	1	100
S4	250 µL S2	1	50
S5	100 µL S2	1	20
S6	100 µL S3	1	10
S7	100 µL S4	1	5
S8	100 µL S6	1	1

160 µL of mobile phase was pipetted into dark tubes. 40 µL of the appropriate standard solution was added and vortexed for 30 seconds. 120 µL was transferred into HPLC glass vials with inlets. Until injection the vials were kept at 4 °C in the dark, otherwise bile pigments would decompose. Standards were frozen at -80 °C until use on analyzing days. At least every 3 – 4 weeks, standards were prepared new, when the highest peak started loosing area (<90%).

Figure 6 shows a HPLC chromatogram of a standard solution with a concentration of 100 µmol/L.



	Retention time	***Area***	**Area Percent**
IIIα-bilirubin	10.376	123289	3.338
IXα-bilirubin	10.941	3426320	92.756
XIIIα-bilirubin	11.560	144312	3.907
Totals		3693921	100.00

Figure 6. HPLC chromatogram of a standard solution [100 µmol/L].

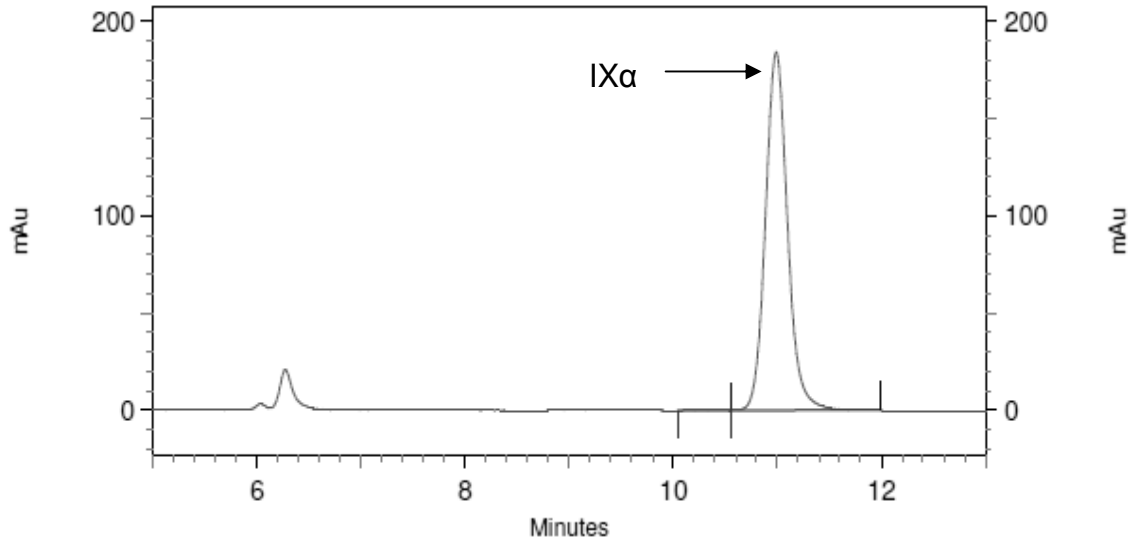
3.2.3 Sample preparation

Blood samples were collected by venipuncture at the AKH in Vacuette® tubes between 8 and 10 am each day and stored in a dark cool place. Samples were transported to university and centrifuged at 3000 RPM for 10 minutes at 4 °C to separate serum from other blood components.

Serum from a healthy person served as a control. The bilirubin level of this control was 4.57 µmol/L and aliquot samples were stored at -80 °C. On days of blood collection a control aliquot was defrosted and analyzed with HPLC together with the samples of the study subjects. The serum of each sample was transferred into dark tubes and remaining serum was fumigated with nitrogen and frozen at -80 °C. 160 µL of mobile phase were put into dark tubes, 40 µL of serum was added and vortexed for 30 seconds. These tubes were centrifuged at 14000 RPM for 5 minutes at 4 °C. After that, 120 µL of the supernatant was

transferred into HPLC glass vials with inlets. Until injection, vials were kept at 4 °C in the dark, to prevent decomposing of the bile pigments.

Humans show only the IXα-bilirubin isomer on HPLC chromatograms. Figure 7 shows the chromatogram of a GS subject with a unconjugated bilirubin concentration of 74.65 µmol/L.



	Retention time	***Area***	**Area Percent**
IIIα-bilirubin	-	-	-
IXα-bilirubin	10.984	2730669	99.823
XIIIα-bilirubin	-	-	-
Totals		2735512	100.00

Figure 7. HPLC chromatogram of a GS subject [74.65 µmol/L].

3.2.4 Reproducibility

The coefficient of variation for this method was tested with the control serum and calculated as shown in the equation below with Microsoft Excel. The intraday variation after 10 control samples was 1.57%. The interday variation throughout the whole analysis from April to September was 5.71%.

Coefficient of variation = (standard deviation / average of samples) x 100%

3.2.5 Statistical analysis

The concentration of unconjugated bilirubin of all study subjects was calculated with Microsoft Excel, using the linear plot from May 10, 2010 with $R^2 = 0.9999$ and $y = 36578x$ (Figure 8). Average, standard deviation and coefficient of variation were calculated with Excel.

Concentration x [µmol/L] = area IXα-bilirubin / k

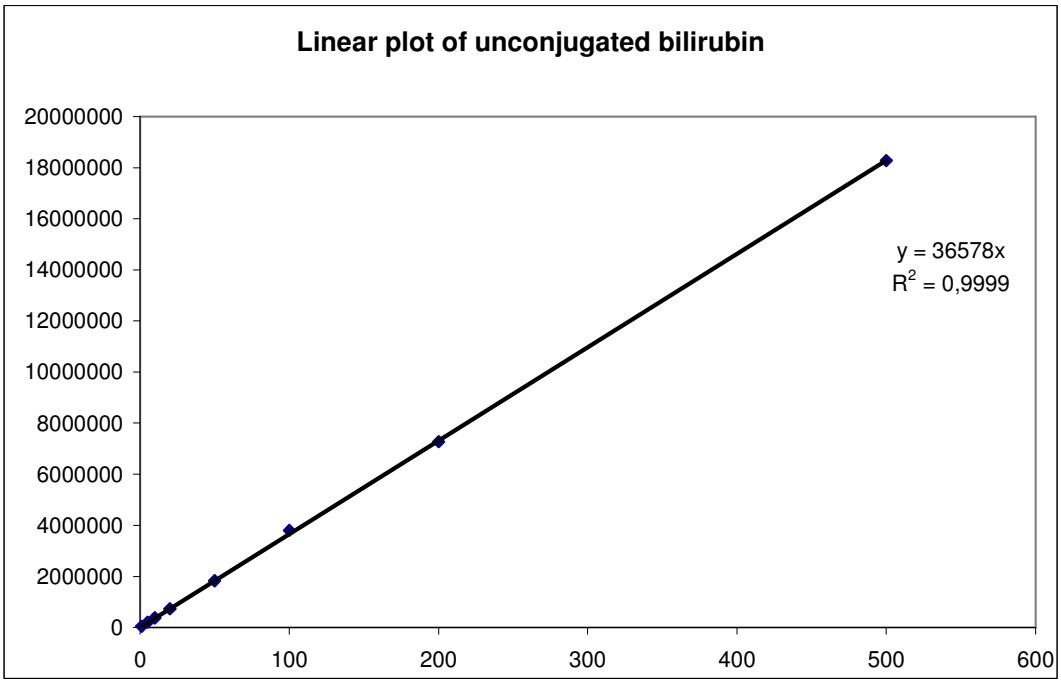


Figure 8. Linear plot of bilirubin used for analysis, done in May 2010.

Data were analyzed with the Statistical Programme for Social Sciences (SPSS) version 17.0. Data were tested for normal distribution by Kolmogorow-Smirnov Test. As unconjugated bilirubin did not show normal distribution, the Mann-Whitney-U-Test was the alternative to the T-test. Bilirubin tertiles were analyzed with the Kruskal-Wallis-H-Test. Data are shown as mean ± standard deviation. $P < 0.05$ was considered as significant and marked with *.

3.3 Cytokinesis-block micronucleus assay

The method of the cytokinesis-block micronucleus (CBMN) cytome assay was adapted from Fenech (2007).

With the CBMN cytome assay chromosome breakage, DNA misrepair, chromosome loss, non-disjunction, necrosis, apoptosis and cytostasis can be measured in human lymphocytes. DNA damages appear in the assay as micronuclei (MN), nucleoplasmic bridges (NPB) and nuclear buds (NBUD). MN are expressed in binucleated (BN) cells that are once-divided due to blocking cytokinesis with cytochalasin-B.

3.3.1 Reagents and equipment

All reagents and the equipment used for the assay are summarized in Table 5 and Table 6.

Table 5. Reagents used in the cytokinesis-block micronucleus assay.

Name	***Manufacturer***	***Factory number***
Cytochalasin-B	Sigma Aldrich	C 6762
Diff-Quick Staining Set	Medion Diagnostics	460.058
DMSO	Sigma Aldrich	D 4540
Entellan	Merck	1.07961.0100
Fetal Bovine Serum “Gold”, heat inactivated	PAA	A 15-152
L-Glutamine	Sigma Aldrich	P 7513
PBS	Sigma Aldrich	D 8537
PHA	PAA	J01-006
RPMI	PAA	E15-039
Sodium pyruvate	Sigma Aldrich	P 2256
Trypanblue	Sigma Aldrich	T 8154

Table 6. Equipment used in the cytokinesis-block micronucleus assay.

Name	***Manufacturer***
Blood collection tubes, Vacuette®	Greiner bio-one
Centrifuge	Eppendorf 5413R
Centrifuge	Thermo Scientific, Megafuge 40
Cytocentrifuge	Thermo Scientific, Cytospin 4
Cell counter	Invitrogen
Centrifuge tubes, Leucosep®	Greiner bio-one
Microscope	Olympus

3.3.2 Sample preparation

3.3.2.1 Blood collection

Blood samples were collected by venipuncture at the AKH in Vacuette® tubes between 8 and 10 am each blood sampling day and stored in a dark cool place. Samples were transported to the university for further preparations.
All steps were done under sterile conditions in the laminar flow and sterile equipment was used until slide centrifugation.

3.3.2.2 Culture medium

The medium for lymphocytes cultivation was prepared by adding 30 mL RPMI + 3.3 mL FBS + 330 µL sodium pyruvate + 330 µL L-glutamine into a sterile 45 mL centrifuge tube.

3.3.2.3 Isolation of lymphocytes

9 mL heparinized blood was transferred carefully with a Pasteur pipette from the blood collecting tube into the ficoll-containing Leucosep® tube. The tubes were centrifuged at room temperature for 15 minutes at 1036 x g without brake.
After centrifugation, a sequence of layers occurred (Figure 9):

From top to bottom:

a) plasma

b) enriched cell fraction (interphase consisting of lymphocytes / PBMCs)

c) separation medium

d) porous barrier

e) separation medium

f) pellet (erythrocytes and granulocytes).

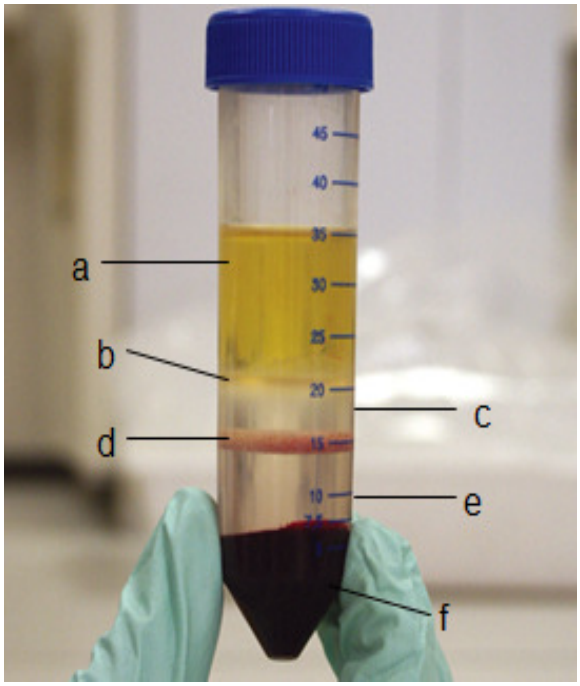


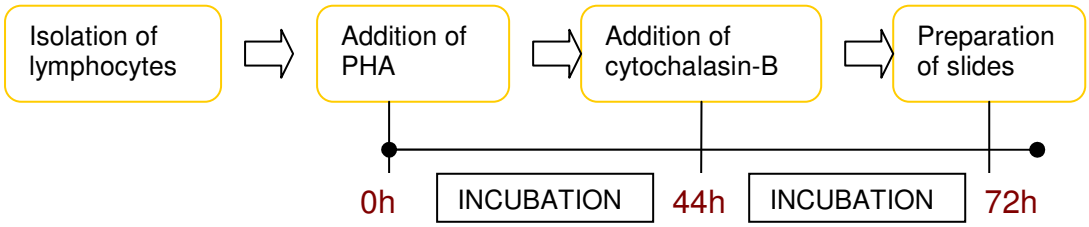
Figure 9. Sequence of layers after centrifugation [Greiner-Bio One, 2011].

From now on, all steps were done on ice. The enriched cell fraction (lymphocytes / PBMCs) was harvested by a sterile plastic Pasteur pipette into a 15 mL centrifugation tube. The cell suspension was washed with 15 mL of cooled PBS and subsequently centrifuged for 10 minutes at 250 x g and supernatant was aspirated by sterile glass Pasteur pipette. This washing step was repeated once and the cell pellet was resuspended in 1 mL of cool PBS.

3.3.2.4 Cell counting

10 µL of diluted cell suspension in PBS were mixed with 10 µL trypanblue, transferred onto a slide and measured by a cell counter. With countess, the cell

number of viable cells was determined (living cells / mL). Tubes with PBS cell-fraction were centrifuged again and medium was added (~1 mL).



3.3.2.5 Incubation and addition of PHA

In total, a volume of 750 µL with a concentration of 10^6 cells/mL was needed. Therefore, adequate µL of cell suspension + RPMI were added in a cell culture tube. Samples were prepared in duplicates (A + B).

By adding 15 µL of PHA the mitotic division of lymphocytes was stimulated. Cells were incubated with lids loose at 37 °C, 5 % CO_2 for exactly 44 hours.

3.3.2.6 Addition of cytochalasin-B after 44 hours

Stock solution of cytochalasin-B in DMSO [600 µg/mL] was diluted to the concentration needed [60 µg/mL] by adding 200 µL stock + 20 µL stock + 1980 µL RPMI.

56.2 µL medium from the supernatant of the culture were removed and replaced with 56.2 µL of diluted cytochalasin-B. Adding cytochalasin-B stopps cell division. Cultures were incubated at 37 °C, 5 % CO_2 for further 28 hours.

3.3.2.7 Harvesting of cells after 72 hours

Slides were labelled, and with filter-cards and cones placed in the cytocentrifuge.

200 µL of the supernatant were taken off and cell suspension was mixed gentle by tapping the tube with the finger. 46 µL DMSO were added and mixed again by finger tapping. 120 µL were transferred directly to the cytocentrifuge cones. From each tube two slides were prepared (a + b).

Cytocentrifugation was performed at room temperature for 5 minutes at 600 RPM, at high acceleration. Subsequently, slides were air-dried for exactly 10 minutes. If slides dry longer, the morphology of cells changes, which makes it difficult to score.

3.3.2.8 Staining of cells

Slides were placed vertically in a dry staining rack and placed in staining solutions:
Diff-Quick fixative: 10 minutes
Diff-Quick solution 1 (red): dipping 10 times
Diff-Quick solution 2 (blue): dipping 8 times.
Keeping the slides in the rack, they were washed gently with tap water and rinsed with pure water, to get rid of dye. Then, slides were placed on paper tissues and dried for about 10-15 minutes. Slides were left to dry completely for at least 30 minutes before putting cover slips on. At maximum, they were left to dry overnight at room temperature.

3.3.2.9 Cover slipping and storage

2-3 drops of entellan were put on the slides next to the spot (not directly on the spot) and a coverslip was placed above it. Slides were dried overnight and finally stored in slide boxes. Per subject, four slides were prepared (Aa, Ab, Ba,Bb).

3.3.3 Scoring of cells

The scoring was done with a light microscope at x1000 magnification.
In the CBMN cytome assay mononucleated, binucleated and multinucleated viable cells, necrotic and apoptotic cells were scored, as well as the frequency of DNA damage biomarkers (MN, NPB and NBUD). In total, 2000 BN cells were

scored per subject, per slide 500 BN. For each slide, the following scheme was obtained:

Until 250 BN cells, all parameters were scored:

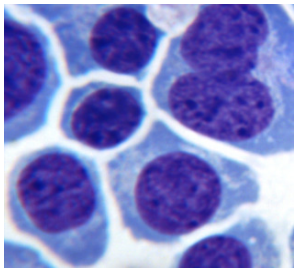
- Mononucleated cells (MONO)
- Binucleated cells (BN)
- Multinucleated cells (MULT)
- Apoptotic cells
- Necrotic cells
- BN with MN
- Total amount of MN in BN
- BN with NPB
- BN with NBUD

After 250 BN, only BNs were considered and counted until 500 BN:

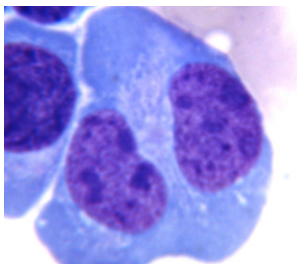
- BN
- BN with MN
- Total amount of MN
- BN with NPB
- BN with NBUD

Slides were scored as described by Fenech. For a detailed description of scoring criteria see Fenech et al. (2003) and Fenech (2007). Pictures below illustrate examples of the cells.

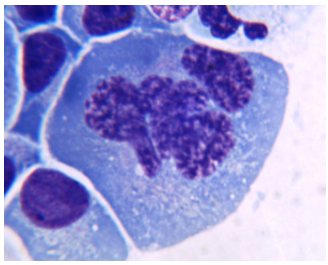
Mononucleated cells



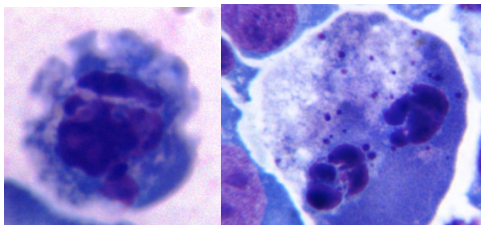
Binucleated cells



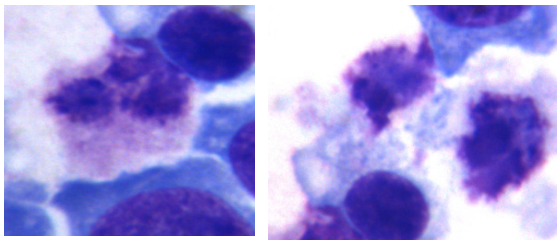
Multinucleated cells



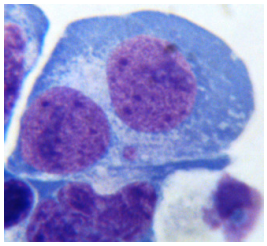
Apoptotic cells

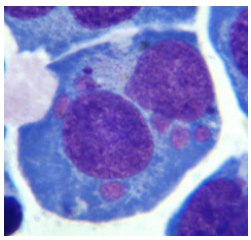


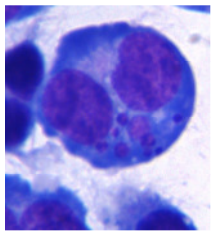
Necrotic cells



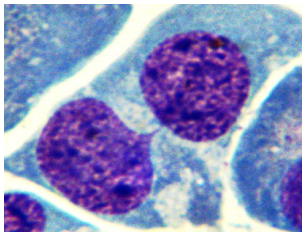
BN with MN

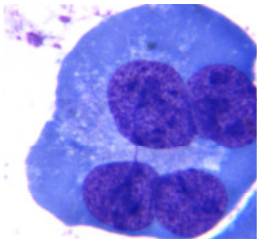




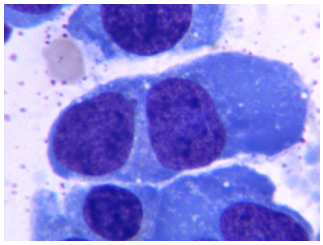


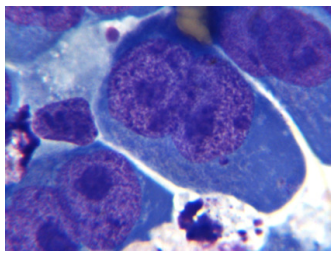
BN with NPB





BN with NBUD





3.3.4 Data calculation

Results of the counting were entered into the scoring sheet and calculated with Microsoft Excel. For every subject, every parameter gives four different data, of which the average can be calculated.

Total amount of cells scored:

MONO+ BN + MULT + Apo + Necro

Frequency of cells in %:

BN / total amount of cells scored * 100
Apo / total amount of cells scored * 100
Necro / total amount of cells scored * 100

Nuclear Division Index (NDI) [Fenech, 2000]:

(MONO + 2xBN + 4xMULT) / viable cells (MONO + BN + MULT)

Nuclear Division Cytotoxicity Index (NDCI) [Fenech, 2000]:

(Apo + Necro + MONO + 2xBN + 4xMULT) / total amount of cells scored

MN, NPB and NBUD per 1000 BN:

BN with MN / BN * 1000
MN / BN * 1000
NPB / BN * 1000
NBUD / BN * 1000

Anticipated results: Fenech (2007) gives the following ranges of values to expect from the CBMN assay:

Frequency of BN: 30–60 %
NDI: 1.3–2.2
Necrotic cells: 0–9 %
Apoptotic cells: 0–7 %
MN per 1000 BN: 0–30
NPB per 1000 BN: 0–10
NBUD per 1000 BN: 0–5

3.3.5 Statistical analysis

Data were analyzed with SPSS 17.0. Data were tested for normal distribution with the Kolmogorow-Smirnov Test. As data were not normally distributed, the Mann-Whitney-U-Test was the choice. When more than two groups were compared, the Kruskal-Wallis-H-Test was used. $P < 0.05$ was considered significant and marked with *.

4 RESULTS and DISCUSSION

4.1 Bilirubin analysis and validation

In total, 100 subjects, including 70 males and 30 females, completed the study. On average, the mean age of the population was 31.6 ± 11.8 years, with a range between 20 and 70 years. The average unconjugated serum bilirubin concentrations and averages are shown in Table 7.

Table 7. Average concentrations and ranges of unconjugated bilirubin in the study population.

	Number	***UCB [µmol/L]***	***Ranges of UCB [µmol/L]***
Total study population	100	20.44 ± 15.14	4.10 – 73.53
Men	70	23.05 ± 16.07	4.28 – 73.53
Women	30	14.34 ± 10.65	4.10 – 41.21

Unconjugated serum bilirubin from the study subjects was determined by HPLC. The control serum was obtained from a healthy person. The interday variation showed the mean of the control serum values measured on each blood sampling day (14 days in total). This coefficient should illustrate stability of the method and of the frozen samples. For the intraday variation a serum sample was analyzed 10 times to show quality and precision of the method. Interday and intraday variations are presented in Table 8.

Table 8. Interday and intraday variation of unconjugated bilirubin.

	Number	***Mean [µmol/L]***	***SD***	***CV (%)***
Interday variation	14	4.58	0.26	5.71
Intraday variation	10	5.08	0.08	1.57

The control serum was filled in dark cups and frozen immediately at -80°C and one cup was thawed for each analyzing day. Figure 10 shows the good stability

of the frozen samples throughout the whole analysis, lasting from April to October. Concentrations ranged between 3.98 and 4.99 µmol/L.

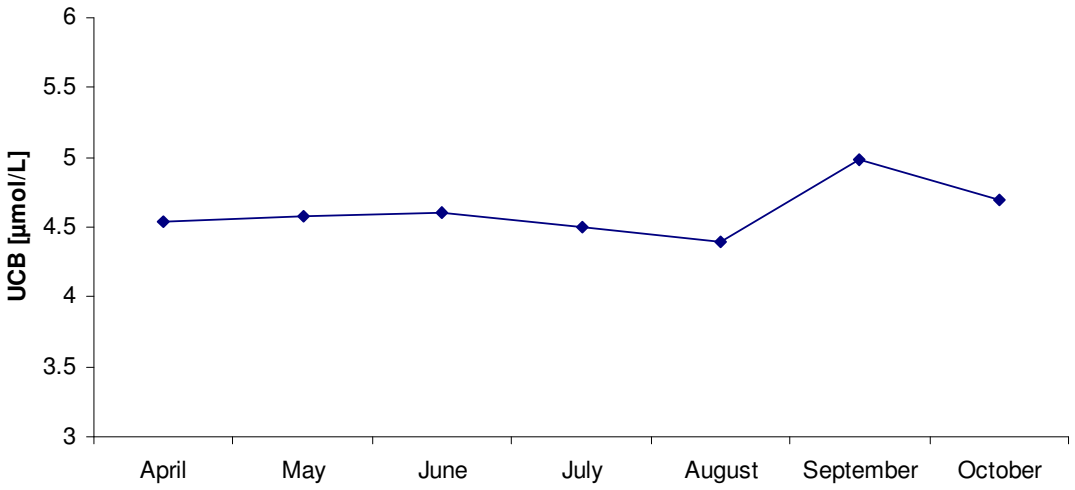


Figure 10. Concentrations of serum control sample over six months.

Although bilirubin is unstable and sensitive to light, these results show that a careful treatment (dim light, fast procedure, frozen immediately) can prevent bilirubin from degradation for a quite long time. Concentrations stayed relatively stable enabling a repeat of the analysis even after some months.

4.2 Micronucleus assay

Data from the CBMN assay were collected from 98 subjects (two values were excluded from the statistical analysis because of extreme MN values).
The average frequencies of MN, NPB and NBUD in the study population were 11.8 ± 9.1; 1.3 ± 1.3 and 2.9 ± 2.2. These values were in the ranges of the anticipated results suggested by Fenech (2007) and cell counting was based on the instructions from the HUMN project [Fenech et al., 2003]. Therefore, the data can be regarded as highly valuable and comparable to other projects.

In the present study, research was focused on the influence of unconjugated bilirubin on DNA. It was proposed that people, affected by the Gilbert's syndrome and therefore having elevated bilirubin concentrations, have less DNA damage (MN, NPB and NBUD) than persons with normal bilirubin concentrations. The population was divided into three similar groups (tertiles), based on their HPLC determined unconjugated serum bilirubin concentrations. The following results compare these three groups.

Data are presented in box plot diagrams. The box includes the average 50% of all data. The length of the box corresponds to the interquartile range. The line in the box represents the median and gives information about the skewness. The lines outside of the box are called whisker and describe data outside the 50%. Dots display outliers, stars show extreme values.

Table 9 summarizes the results of the CBMN assay in three bilirubin groups. There were no significant differences found between the groups. MN values did hardly differ and NPB decreased slightly with higher bilirubin concentrations. NBUD showed and increasing tendency, which is inconsistent with the hypothesis that UCB can protect from DNA damage. This trend is displayed in Figure 11.

Table 9. Chromosomal damage measured by the CBMN assay of study subjects in bilirubin tertiles.

	Tertile 1: 4.10–11.12 µmol/L	***Tertile 2: 11.29–20.69 µmol/L***	***Tertile 3: 20.86–73.53 µmol/L***	***pValue***
Number	33	34	33	-
Age [years]	30.64 ± 11.25	29.94 ± 10.50	34.39 ± 13.34	0.202
MN total in BN/1000 BN	11.03 ± 9.11	12.24 ± 9.05	12.22 ± 9.30	0.789
BN with MN/1000 BN	11.72 ± 12.05	10.67 ± 6.74	10.68 ± 7.21	0.896
BN with NPB/1000 BN	1.34 ± 1.33	1.32 ± 1.27	1.09 ± 1.38	0.365
BN with NBUD/1000 BN	2.34 ± 1.45	3.00 ± 2.20	3.37 ± 2.76	0.576

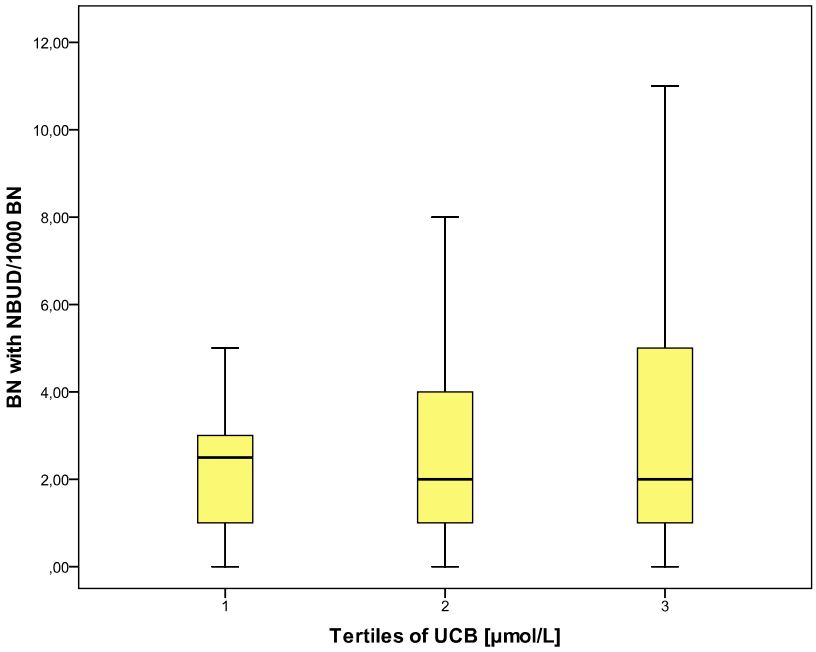


Figure 11. NBUD in BN/1000 BN in bilirubin tertiles.

Although the mean values of NBUD tended to increase with higher bilirubin concentrations, the median in the boxplot is decreased. The variability was much larger in tertile 3 than in tertile 1, suggesting that there were subjects with relatively inconsistent NBUD values.

NBUD are assumed to represent amplified DNA, which is removed during the S phase of the cell cycle, but they may also arise after exposure to γ-irradiation. Most NBUD are formed from interstitial or terminal acentric fragments, which

might refer to nuclear membranes of DNA left in the cytoplasm, however, the process of NBUD formation is not fully understood yet [Fenech et al., 2011]. Therefore, only a few studies presented data of NPB and NBUD so far, making it almost impossible to interpret these findings. Generally, it is difficult to compare total values of the CBMN assay, as there are a lot of influencing factors (e.g. counters, conditions). But most studies used the same method, previously described by Fenech (2007), therefore tendencies can be evaluated quite well.

Coskun et al. (2011) compared CBMN frequencies of pesticide exposed farmers to controls. The total frequencies for NBUD were 0.31 ± 0.13 for controls and 1.22 ± 0.21 for farmers, suggesting higher DNA damage in the exposed subjects. However, our mean values of NBUD increased with higher bilirubin concentrations, which seems to be inconsistent with our hypothesis that DNA is protected by higher bilirubin levels. Consequently, more research on the effects of bilirubin in relation with the micronucleus assay is needed. To the best of our knowledge, only one study (Grant et al., 2004) focused on bilirubin and MN frequencies. However, this group did not measure bilirubin concentrations, but detected the UGT1A1 polymorphism, which causes elevated bilirubin levels. Moreover, they used a slightly different method. Given that, they could not show a protecting effect of bilirubin either. Grant et al. suggested that bilirubin might not work as an antioxidant in lymphocytes or that a higher amount of oxidative stress would be needed, to be able to measure a protecting outcome. Additionally, 48 of the 63 subjects were smokers, and smoking is assumed to lower the antioxidant effect of bilirubin [Schwertner, 1998], so this might have influenced the results from Grant et al. In our study, smokers were excluded to avoid this effect, but there was still no protective influence on DNA measurable. However, it might be possible to measure the DNA protecting properties of antioxidants with the CBMN assay. In a study by Fenech et al. (2005) the effect of dietary intake on MN formation was investigated in 190 healthy subjects. Five micronutrients could be identified to have a decreasing potential on DNA damage. Vitamin E, retinol, folate, nicotinic acid and calcium could reduce MN

frequencies. As vitamin E is an antioxidant of high potential, this study showed that antioxidants had a positive effect on DNA stability. It was suggested that vitamin E might protect DNA from mutation triggered by reactive oxygen species. Bilirubin is assumed to be an antioxidant as strong as vitamin E [Stocker et al., 1987], therefore, we could have expected a lower DNA damage in the Gilbert's syndrome group. Hence, the present results showed that UCB did not have a significant effect on the formation of DNA damage. However, this was the first study that examined a relationship of bilirubin concentrations and DNA damage, consequently more research is needed.

4.2.2 Influencing factors

4.2.2.1 Age

- **Age in tertiles**

Table 10. DNA damage of study subjects in age tertiles.

	Tertile 1: 20-24 years	***Tertile 2: 25-32 years***	***Tertile 3: 33-70 years***	***pValue***
Number	32	32	34	-
UCB [µmol/L]	18.96 ± 14.29	19.44± 13.88	22.82 ± 17.14	0.690
MN total in BN/1000 BN	7.40 ± 3.17	9.68 ± 7.59	18.24 ± 10.71	**0.000*** **
BN with MN/1000 BN	6.90 ± 3.03	8.56 ± 5.22	17.20 ± 11.64	**0.000*** **
BN with NPB/1000 BN	1.06 ± 1.19	1.40 ± 1.54	1.29 ± 1.22	0.479
BN with NBUD/1000 BN	2.06 ± 1.50	2.90 ± 1.97	3.70 ± 2.71	**0.024***

Significant differences were found in MN and NBUD (Table 10); these results are displayed in boxplots (Figure 12 and Figure 13). Total MN and BN with MN in tertile 3 were significant higher than tertile 2 and 1 (3>2>1), in NBUD tertile 3 was only significant with tertile 1.

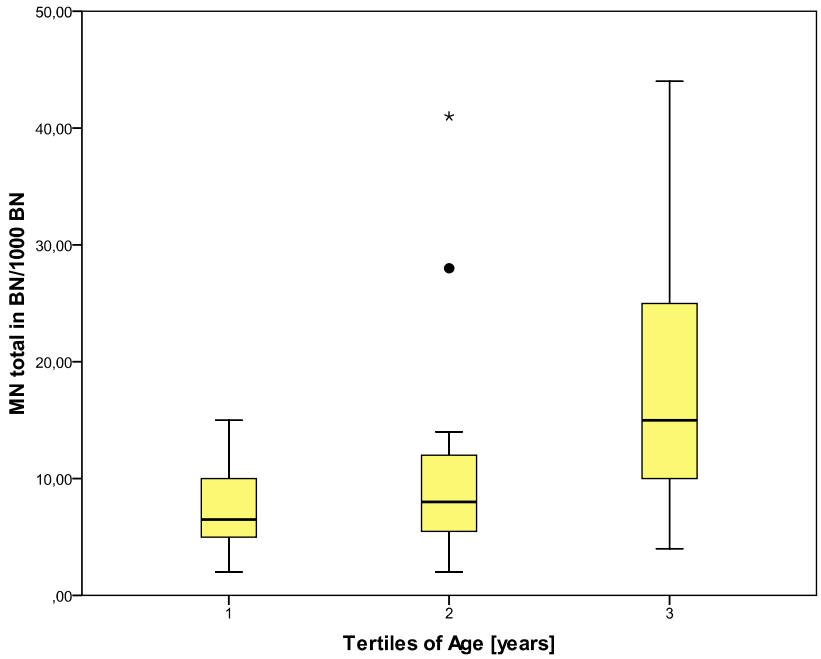


Figure 12. Total MN in BN/1000 BN in age tertiles.

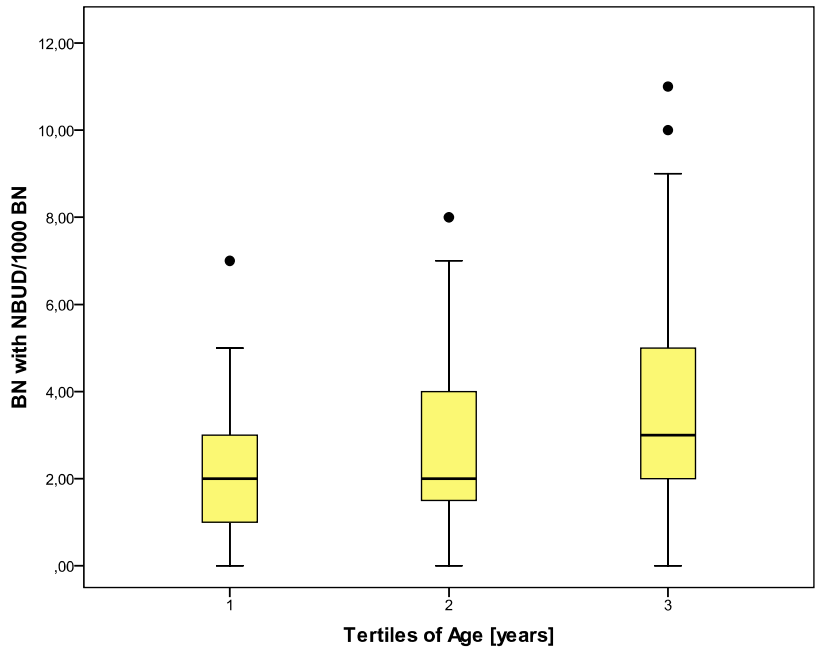


Figure 13. NBUD in BN/1000 BN in age tertiles.

Age was highly significant with MN and NBUD. With increasing age, more MN and NBUD were found, indicating an influence of aging. Additionally, the median increased with age, both in MN and NBUD and in both cases the variability was largest in the oldest group. Especially, MN showed a wide

variability in tertile 3 compared to the younger ones, which suggests that there were some people with quite high MN frequencies as well as with low ones.

The increase of MN with age is in agreement with other authors. Wojda et al. (2007) described a higher frequency of MN in older people. Bonassi et al. (2001) reported that people aged over 40 years had more MN. Mateuca et al. (2006) showed that MN frequencies rose with age. Garaj-Vrhovac et al. (2008) showed that subjects under 30 years had the lowest MN median values and frequencies were increasing from then on.

MN increase with age for many reasons, an unhealthy lifestyle can be named as a general cause, but also the exposure to toxic compounds. A poor diet, stress, sickness, alcohol and physical activity can also cause elevated chromosomal damage [Fenech & Bonassi, 2011]. Although a few studies showed data for NPB and NBUD [Coskun et al., 2011; Garaj-Vrhovac et al., 2008], no influence of age was presented in these markers.

- **Age in two groups and in bilirubin tertiles**

Table 11. DNA damage of study subjects in two age groups and in bilirubin tertiles.

		20-29 years	***30-70 years***	***pValue***
Tertile 1	**Number**	22	10	-
	UCB [µmol/L]	7.66 ± 2.11	7.35 ± 1.76	0.709
	MN total in BN/1000 BN	7.59 ± 3.73	19.44 ± 12.74	**0.002****
	BN with MN/1000 BN	7.09 ± 3.54	21.90 ± 17.43	**0.001*****
	BN with NPB/1000 BN	1.32 ± 1.58	1.40 ± 0.51	0.130
	BN with NBUD/1000 BN	2.04 ± 1.55	3.00 ± 0.94	0.062
Tertile 2	**Number**	21	13	-
	UCB [µmol/L]	16.09 ± 3.20	14.31 ± 3.05	0.184
	MN total in BN/1000 BN	9.14 ± 5.46	17.23 ± 11.47	**0.019***
	BN with MN/1000 BN	8.57 ± 5.35	14.07 ± 7.53	**0.020***
	BN with NPB/1000 BN	1.47 ± 1.47	1.07 ± 0.86	0.683
	BN with NBUD/1000 BN	2.52 ± 1.88	3.77 ± 2.52	0.198

Tertile 3	**Number**	16	16	-
	UCB [µmol/L]	38.87 ± 11.98	38.07 ± 13.77	0.705
	MN total in BN/1000 BN	6.81 ± 2.71	17.62 ± 10.44	**0.001*** **
	BN with MN/1000 BN	6.43 ± 2.47	14.93 ± 7.92	**0.001*** **
	BN with NPB/1000 BN	0.93 ± 1.06	1.25 ± 1.65	0.842
	BN with NBUD/1000 BN	2.50 ± 1.63	4.25 ± 3.37	0.198

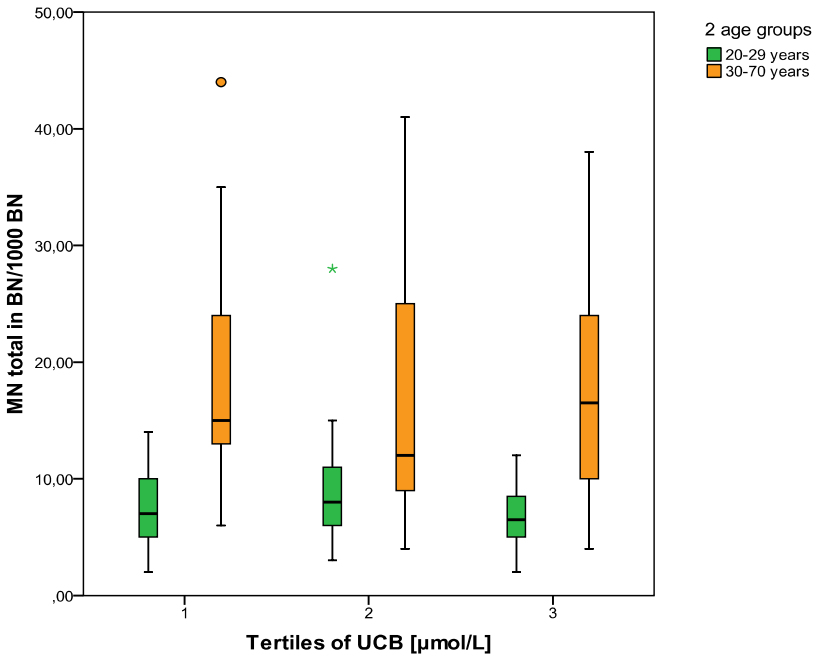


Figure 14. Total MN in BN/1000 BN in two age groups and in bilirubin tertiles.

MN frequencies were significantly higher in the oldest group (see Table 11 and Figure 14). NPB and NBUD tended to be higher as well, but not with statistical significance. While NBUD were significant in age tertiles (Table 10 and Figure 13), this effect was not measurable in two age groups (Table 11). In this analysis, data were also split in bilirubin tertiles, but there was no significant effect of bilirubin. However, the mean values of MN frequencies were lower in tertile 2 and 3 in the older group than in tertile 1, while NBUD were highest in the old group with high bilirubin concentrations. These results might indicate that an unconjugated bilirubin concentration of 11.29 µmol/L and higher can prevent from chromosomal damage. Further observations in an older population

with Gilbert's syndrome are needed to clarify whether a longer exposure to bilirubin has a protective effect.

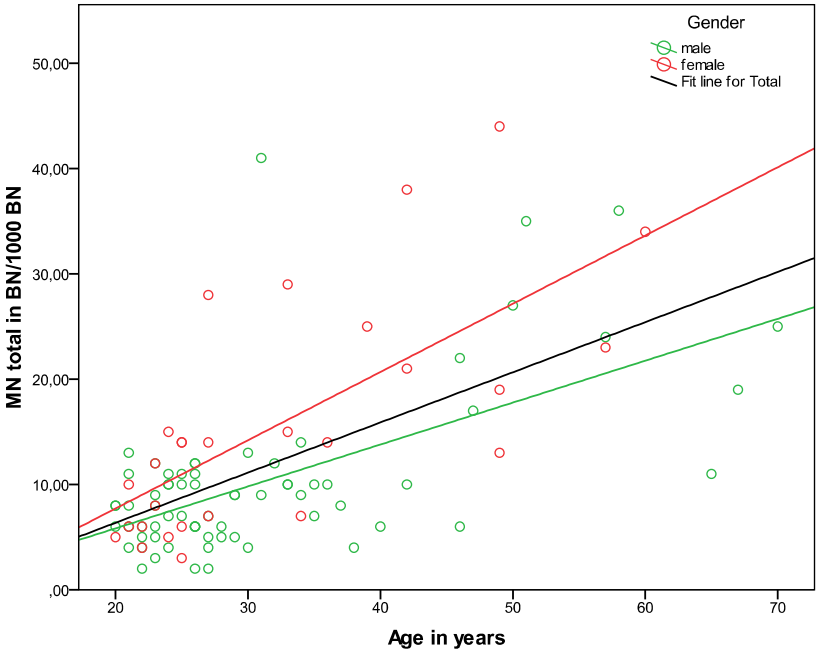


Figure 15. Correlation of total MN in BN/1000 BN and age.

Strong correlations were found in age and MN (Figure 15), in the whole study population (r=0.524; p=0.000) and in both genders (men: r=0.424; p=0.000; women: r=0.729; p=0.000). This result indicates that MN increased with age which is in agreement with the literature (see discussion before).

4.2.2.2 Gender

Table 12. DNA damage of study subjects in men and women.

	Men	***Women***	***pValue***
Number	70	30	-
UCB [µmol/L]	23.05 ± 16.07	14.34 ± 10.65	**0.002****
Age [years]	31.13 ± 11.71	32.83 ± 12.09	0.630
MN total in BN/1000 BN	10.29 ± 7.76	15.68 ± 10.93	**0.012***
BN with MN/1000 BN	8.98 ± 5.60	15.86 ± 12.72	**0.004****
BN with NPB/1000 BN	1.27 ± 1.42	1.20 ± 1.05	0.723
BN with NBUD/1000 BN	2.91 ± 2.19	2.89 ± 2.33	0.924

The influence of gender was significant in UCB and MN frequencies (Table 12).

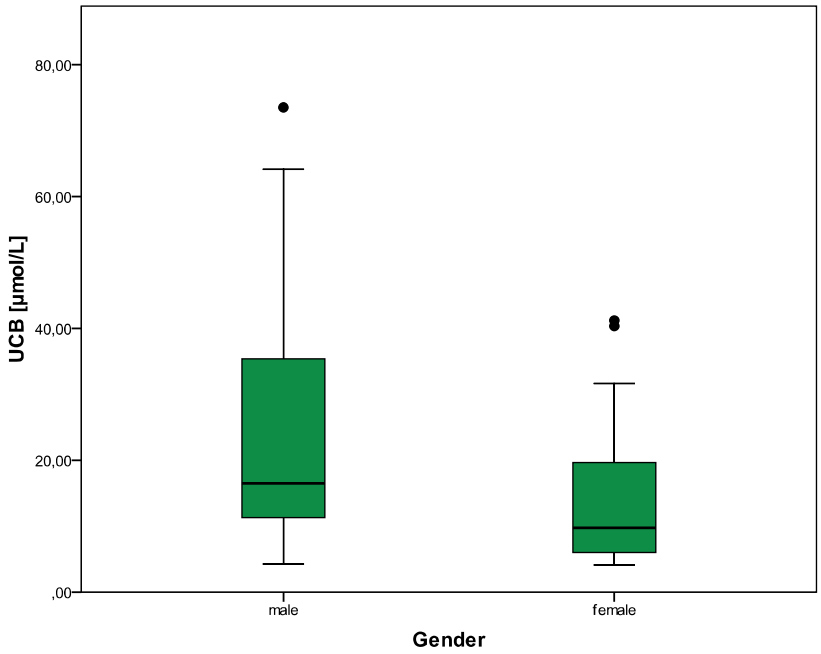


Figure 16. Bilirubin concentrations in men and women.

UCB was significantly higher in men than in women (p=0.002). There was a greater variability in men than in women, which might be due to the fact that 70 men but only 30 women were included in the trial (Figure 16). Subjects with high bilirubin concentrations (>17.1 µmol/L) were diagnosed with Gilbert's syndrome. It is known that this disease affects more men than women (12.4 vs. 4.8%), and mean serum bilirubin concentrations are significantly higher in men. One explanation for this observation suggests that men have more bilirubin per kilogram body weight and they are generally heavier than women. Another explanation is the different hormonal balance, causing androgen steroid inhibition of bilirubin enzymatic glucuronidation [Radu & Atsmon, 2001].

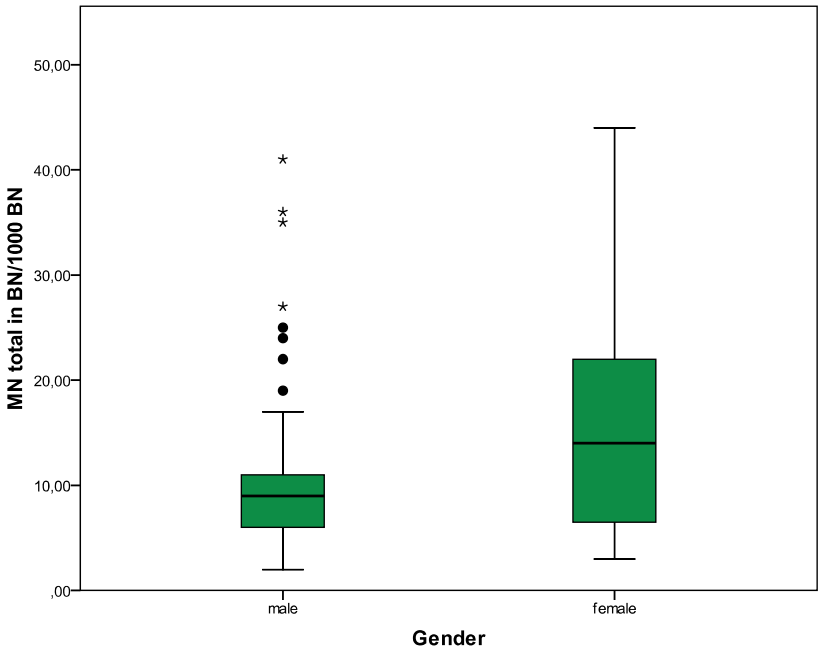


Figure 17. Total MN in BN/1000 BN in men and women.

MN frequencies were significantly higher in women ($p = 0.012$; see Figure 17), which is in agreement with other authors [Bonassi et al., 2001; Costa et al., 2007; Mateuca et al., 2006; Wojda et al., 2007]. It was assumed that the X chromosome gets lost as MN more often than other chromosomes. As women have two X chromosomes and men have only one, it is believed that this might be the reason for higher MN frequencies in women. [Fenech & Bonassi, 2011]. However, this should not indicate that women generally have a higher cancer risk than men.

4.2.2.3 Vitamins

- **Folic acid**

Table 13. DNA damage of study subjects in folic acid tertiles.

	Tertile 1: 38.90- 72.80 ng/mL	***Tertile 2: 74.10- 152.40 ng/mL***	***Tertile 3: 157.10- 510.40 ng/mL***	***pValue***
Number	31	32	30	-
UCB [µmol/L]	21.68 ± 13.31	21.13 ± 17.06	19.53 ± 15.96	0.395
Age [years]	34.39 ± 11.01	35.03 ± 13.95	24.66 ± 6.75	**0.000*** **
MN total in BN/1000 BN	13.19 ± 9.99	13.03 ± 9.50	8.76 ± 5.64	0.102
BN with MN/1000 BN	11.22 ± 7.12	13.22 ± 11.83	8.13 ± 4.83	0.109
BN with NPB/1000 BN	1.39 ± 1.74	1.50 ± 1.07	0.93 ± 1.04	0.053
BN with NBUD/1000 BN	3.55 ± 2.79	3.22 ± 2.12	2.16 ± 1.31	0.092

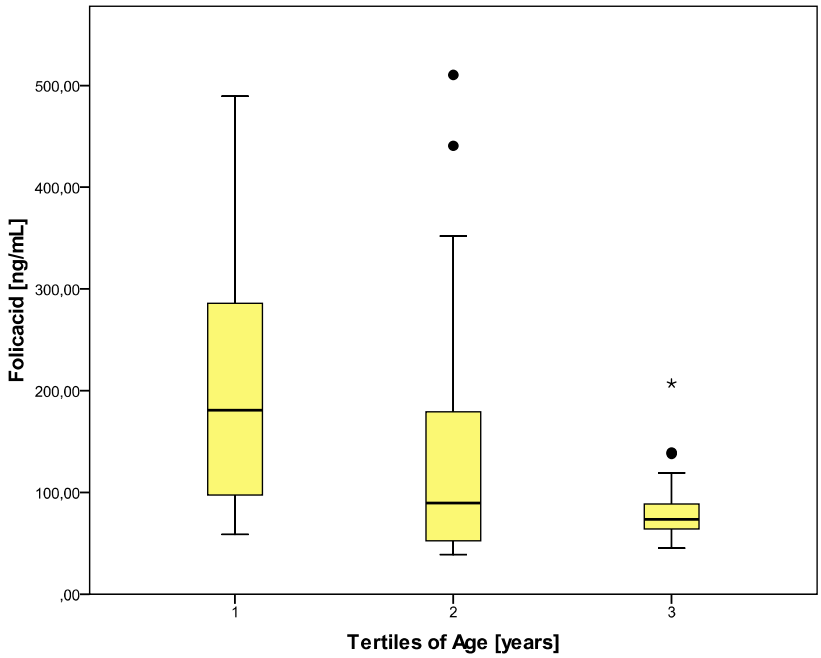


Figure 18. Folic acid concentrations in age tertiles.

As illustrated in Table 13, highest folate concentrations were measured in the youngest age group. To display these results in a boxplot, age tertiles from

above were used. Folic acid concentrations were then classified as follows: tertile 1: 209.05 ± 121.59 ng/mL, tertile 2: 135.89 ± 119.06 ng/mL and tertile 3: 81.78 ± 34.14 ng/mL (p = 0.000). The median decreased with age of the subjects. Also, the variability is very small in the oldest group, suggesting a homogeneous group (Figure 18). Tertile 3 was significant with tertile 2 and 1 (3<2<1).

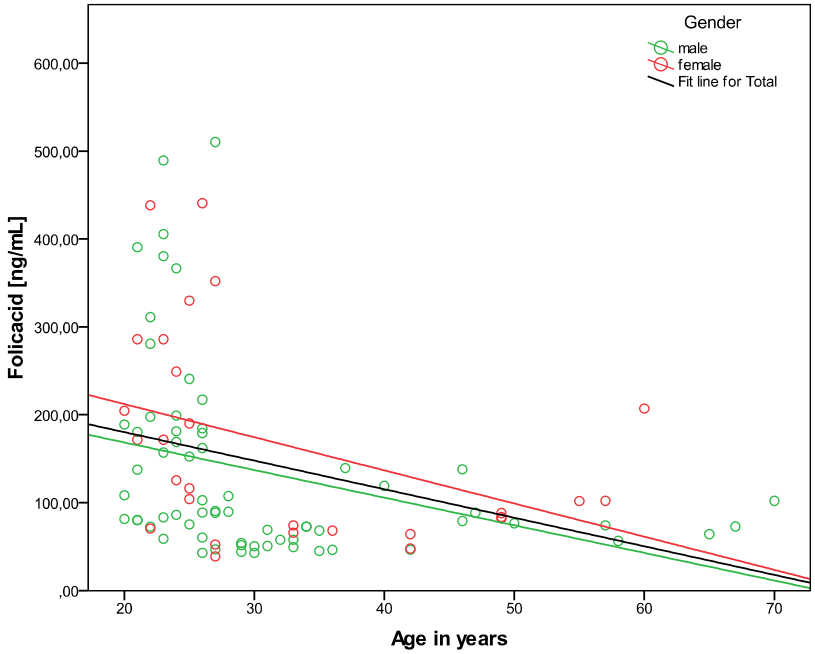


Figure 19. Correlation of folic acid and age.

Folic acid and age correlated significantly (Figure 19) in the whole study population (r = -0.496; p = 0.000), in men (r = -0.522; p = 0.000) and in women (r = -0.467; p = 0.012) in a negative way.

Folic acid also correlated significantly with total MN (Figure 20) in the whole study group (r = -0.221; p = 0.035) and in women (r = -0.497; p = -0.010) negatively. High folic acid concentrations caused a lower MN frequency, suggesting DNA protection by this nutrient.

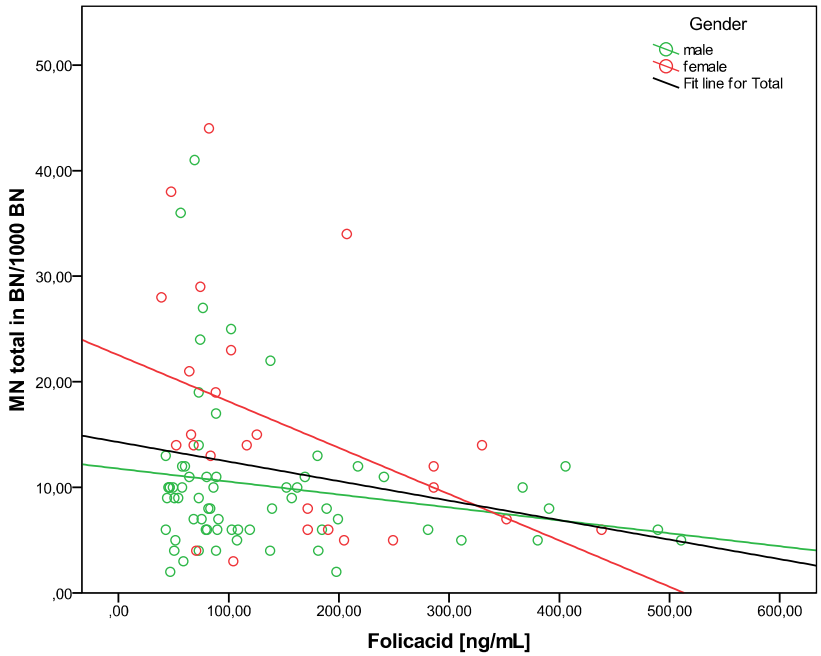


Figure 20. Correlation of total MN in BN/1000 BN and folic acid.

Folate intakes >200 µg/day were recommended to secure chromosomal stability [Fenech et al., 2005]. The D-A-CH (Germany-Austria-Switzerland) reference values even recommend 400 µg/day [Elmadfa et al., 2009]. No significances were found with DNA damage markers, but there was a trend with less chromosomal damage in the group with highest folic acid concentrations. In tertile 3, MN, NPB and NBUD were lowest, suggesting a DNA protecting effect of folic acid. As the group with high folic acid concentrations was also the youngest, it is hard to say if the influence of age or folic acid was the dominant one. However, it is commonly known that folic acid is an important nutritive substance for cell division and DNA stability. Deficiencies cause hypomethylation of DNA leading to incorporation of uracil instead of thymine in the DNA, leading to DNA damage [Fenech, 2001]. In another study by Fenech et al. (2005) folate had been identified as one of five micronutrients exerting a lowering effect on MN formation. Given that, our results showed that folic acid protects the DNA.

- **Vitamin B12 (cobalamin)**

Table 14. DNA damage of study subjects in vitamin B12 tertiles.

	Tertile 1: 1.10-1.40 pM/L	***Tertile 2: 1.50-2.10 pM/L***	***Tertile 3: 2.20-3.90 pM/L***	***pValue***
Number	37	28	29	-
UCB [µmol/L]	1.22 ± 0.98	1.32 ± 0.84	1.08 ± 0.85	0.176
Age [years]	33.51 ± 13.30	31.24 ± 10.99	28.53 ± 10.35	0.156
MN total in BN/1000 BN	13.14 ± 7.40	10.25 ± 7.23	10.96 ± 11.37	**0.016***
BN with MN/1000 BN	13.38 ± 10.21	9.03 ± 5.15	9.27 ± 8.76	**0.006****
BN with NPB/1000 BN	1.70 ± 1.37	1.14 ± 1.53	0.86 ± 0.87	**0.003****
BN with NBUD/1000 BN	3.89 ± 2.19	2.86 ± 2.24	1.86 ± 1.77	**0.000*****

In contrast to previously discussed folic acid, vitamin B12 showed significances with MN, NPB and NBUD (Table 14). All three markers were lower at high vitamin B12 concentrations. Medians were increasing with higher concentrations (Figure 21, Figure 22 and Figure 23). These results suggest a high influence of this vitamin on DNA stability and protection.

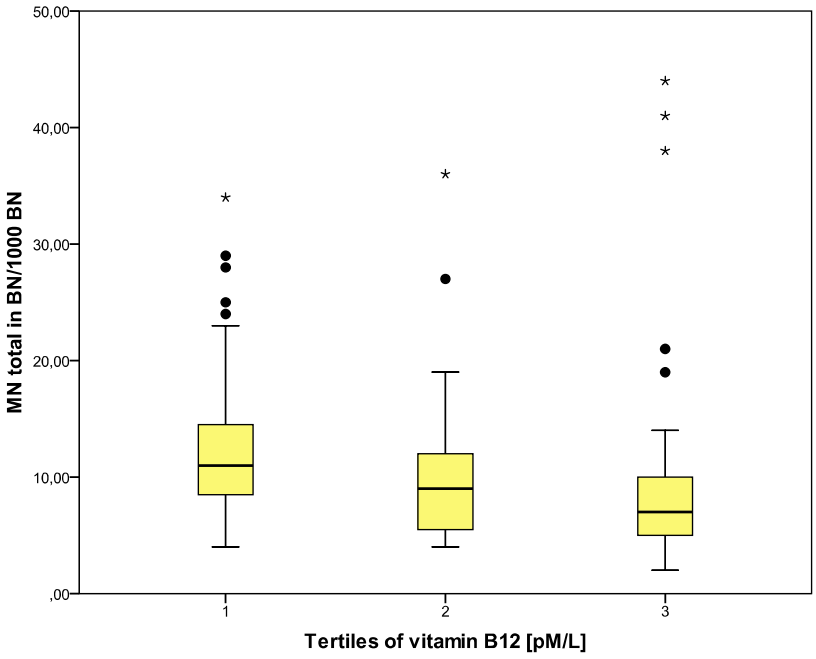


Figure 21. Total MN in BN/1000 BN in vitamin B12 tertiles.

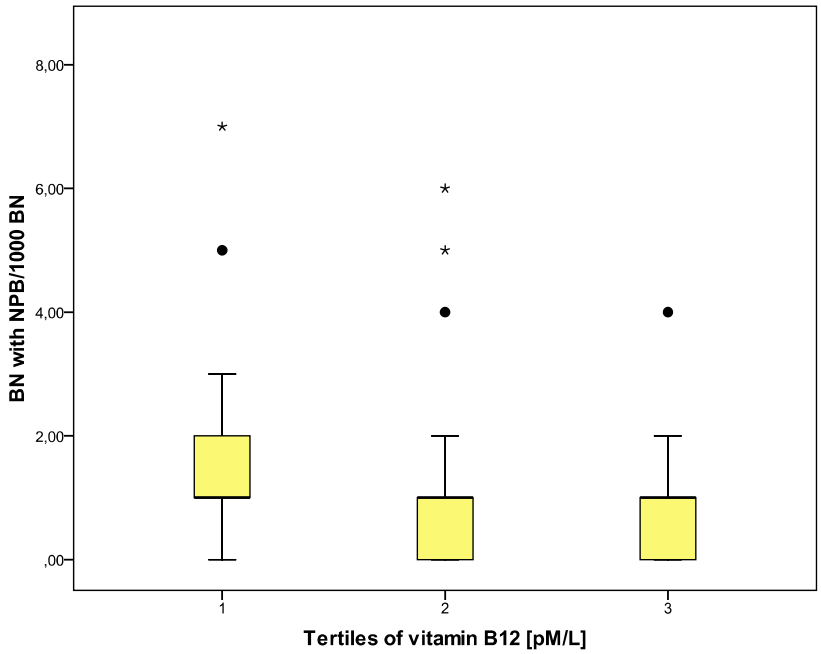


Figure 22. NPB in BN/1000 BN in vitamin B12 tertiles.

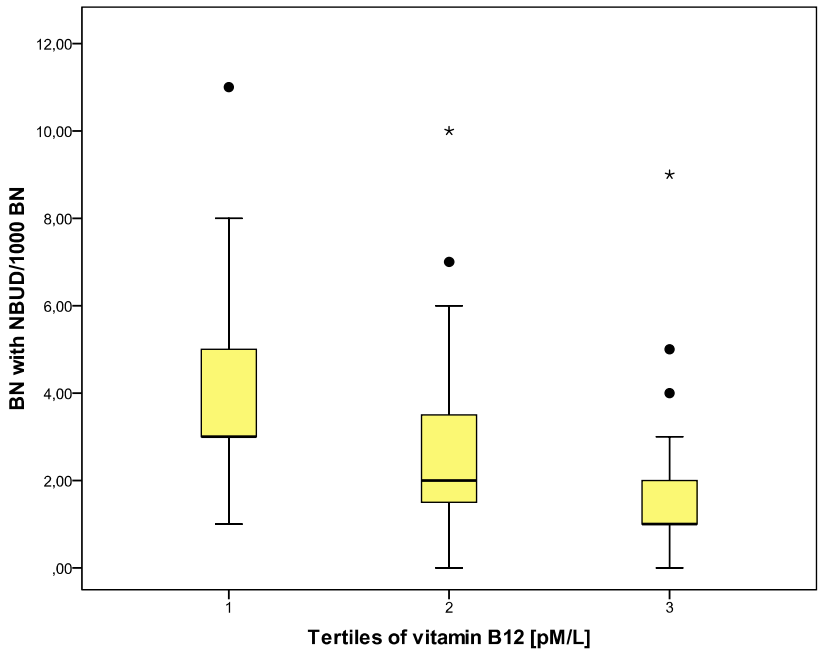


Figure 23. NBUD in BN/1000 BN in vitamin B12 tertiles.

In NPB and NBUD tertile 3 was significant with tertile 1.

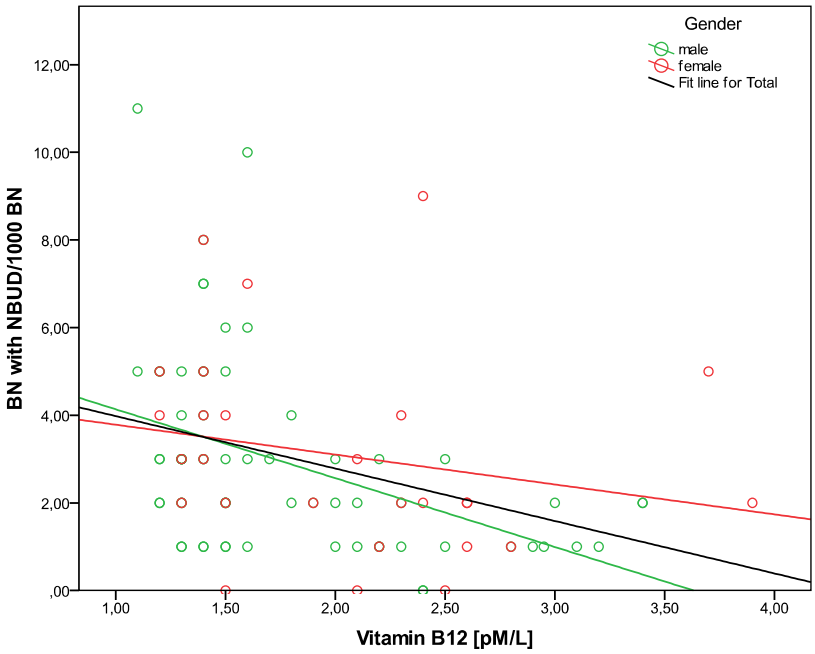


Figure 24. Correlation of NBUD in BN/1000 BN and vitamin B12.

Vitamin B12 correlated significantly with NBUD (Figure 24) in a negative way in the whole study population ($r = -0.452$; $p = 0.000$) and in men ($r = -0.500$; $p = 0.000$). Therefore, we conclude that high concentrations of vitamin B12 prevent from NBUD formation.

Along with folic acid, also vitamin B12 is involved in maintaining DNA stability. Both micronutrients are important for the synthesis of methionine and S-adenosyl methionine (SAM). Vitamin B12 deficiencies and low methionine concentrations reduce SAM synthesis, leading to uracil incorporation into DNA [Fenech, 2001]. According to the Austrian Nutrition Report from 2008, the Austrian population is quite well supplied with this nutrient [Elmadfa et al., 2009]. Several studies measured MN frequencies in relation to folic acid and vitamin B12 [Stopper et al., 2008; Minozzo et al., 2010; Milic et al., 2010]. To our mainly male study population were the results of Milic et al. (2010) most comparable, as in this study, 36 healthy males were investigated. Milic et al. showed that persons with the lowest vitamin B12 concentrations had the highest values of MN, what is exactly what we have found.

4.2.2.4 Influences within DNA damage markers

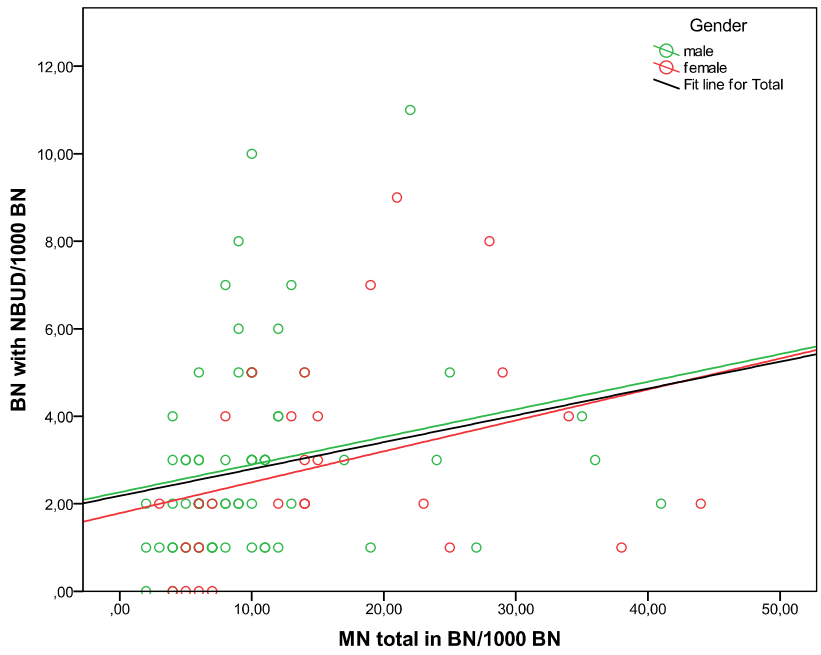


Figure 25. Correlation of total MN in BN/1000 BN and NBUD in BN/1000 BN.

DNA damage markers also showed significant correlations among each other. For example, total MN and NBUD correlated highly significantly (Figure 25) in the whole study population ($r = 0.426$; $p = 0.000$) as well as in men ($r = 0.397$; $p = 0.001$) and in women ($r = 0.489$; $p = 0.008$). Same results for correlations had been shown previously by Coskun et al. (2010) in pesticide exposed farmers. The authors suggested that besides clastogenic and aneugenic effects, pesticides might cause additional gene amplification. However, there is too little information to give assumptions about this relationship. Due to the different origin of chromosomal damage markers the influence of pro-oxidants is still unknown and further investigations are needed. Especially on NBUD and NPB less data are available yet.

4.2.3 Correlations

Table 15. Correlations of all study variables.

	UCB	***Age***	***MN total in BN***	***BN with MN***	***BN with NPB***	***BN with NBUD***	**Folicacid**
Age	0.129 (0.202)	-	-	-	-	-	-
MN total in BN	0.068 (0.509)	0.524 (**0.000*****)	-	-	-	-	-
BN with MN	0.053 (0.607)	0.545 (**0.000*****)	0.990 (**0.000*****)	-	-	-	-
BN with NPB	-0.059 (0.561)	0.083 (0.419)	0.272 (**0.007****)	0.298 (**0.003****)	-	-	-
BN with NBUD	0.103 (0.311)	0.273 (**0.007****)	0.426 (**0.000*****)	0.462 (**0.000*****)	0.545 (**0.000*****)	-	-
Folic acid	-0.125 (0.229)	-0.496 (**0.000*****)	-0.221 (**0.035***)	-0.207 (**0.046***)	-0.074 (0.482)	-0.205 (**0.049***)	-
Vitamin B12	-0.052 (0.618)	-0.196 (0.055)	-0.262 (**0.011***)	-0.288 (**0.005****)	-0.339 (**0.001****)	-0.452 (**0.000*****)	-0.089 (0.392)

MN total, BN with MN, BN with NPB and BN with NBUD are per 1000 BN

Many significant correlations were found (Table 15), especially with folic acid and vitamin B12. No significant correlations were found with UCB.

A number of significant correlations were found either in men or women or in the whole study population (Table 16). Non-significant correlations are not shown.

Table 16. Correlations between different variables in men and women.

Correlation	***Men R (pVal)***	***Women R (pVal)***
Age & MN total in BN/1000 BN	0.424 (**0.000*****)	0.729 (**0.000*****)
Age & folic acid	-0.522 (**0.000*****)	-0.467 (**0.012***)
MN total in BN/1000 BN & folic acid	-0.161 (0.196)	-0.497 (**0.010****)
Vitamin B12 & BN with NBUD/1000 BN	-0.500 (**0.000*****)	-0.365 (0.061)
MN total in BN & BN with NBUD/1000 BN	0.397 (**0.001*****)	0.489 (**0.008****)

Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning.
Albert Einstein

5 CONCLUSIONS

In the present work, the effects of unconjugated bilirubin on oxidative DNA damage were investigated in peripheral blood lymphocytes of 100 healthy individuals. Bilirubin has been reported to have antioxidant properties and could therefore protect from oxidative damage. The study population was divided into three groups, referring to their determined unconjugated bilirubin concentrations, and their DNA damage parameters micronuclei, nucleoplasmic bridges and nuclear buds were compared. However, no statistical differences were found between the groups. Nevertheless, this study shows first results for elevated serum bilirubin levels and the micronucleus assay, and can be regarded as a novel finding. The collected data are highly valuable and can be an impulse for other researchers.

We could show that micronuclei were increased with age and that women had higher micronuclei frequencies than men. Both findings are in line with the literature. The average age of the present study population was 32 years, for further investigations we would suggest an older population, as a protecting effect of bilirubin might be noticeable by lengthen the exposure time of UCB. There was a hint of less DNA damage in older participants with high bilirubin concentrations, however, to prove that fact, more studies need to be done.

Moreover, we could show dietary influences on DNA stability. Folic acid and vitamin B12 are involved in DNA metabolism, as deficiencies lead to the incorporation of uracil. In our study, especially vitamin B12 was shown to have protective properties on DNA stability. Individuals with high concentrations of this micronutrient showed significantly less DNA damage than persons with low vitamin B12 levels. These vitamins have been earlier reported to be protective.

As other antioxidants (e.g. vitamin E) have been reported to show a protective effect on DNA, measured by the cytokinesis-block micronucleus assay [Fenech et al., 2005], it might be possible to find a protective effect of bilirubin. Further research needs to be done to get more insights on this strong and potent antioxidant.

6 SUMMARY

The aim of the present study, a project funded by the FWF-Austrian Science Fund (project number P21162), was to investigate the physiological relevance, especially the antioxidative and anticancer effect of unconjugated bilirubin in humans.

Therefore, persons with mildly elevated unconjugated bilirubin concentrations, diagnosed with Gilbert's syndrome, as well as healthy individuals as controls, were recruited. In total, 100 subjects completed the study and gave blood samples. Unconjugated serum bilirubin was determined by HPLC. This assessment was the basis for the allocation of the subjects into different groups. To measure DNA damage, the cytokinesis-block micronucleus assay was conducted, a state of the art method for detecting chromosomal DNA damage in humans. Lymphocytes were isolated from the blood samples, treated and fixed on slides. Cells were examined under a microscope and scored. In these binucleated cells, micronuclei, nucleoplasmic bridges and nuclear buds can occur, which are DNA damage markers and indicate chromosomal rearrangement.

Bilirubin has been reported to have antioxidant properties, therefore it was hypothesized that individuals with elevated bilirubin concentrations show less DNA damage markers than controls. However, the hypothesis could not be confirmed, there were no significant differences between the groups, concerning bilirubin concentrations. But we could show that micronuclei increase with age and that women have more micronuclei than men. This might indicate that the study population was too young (average 32 years) to show significant differences. Another result of the study was that subjects with high vitamin B12 concentrations had less DNA damage, confirming that this vitamin is involved in the DNA metabolism.

7 ZUSAMMENFASSUNG

Ziel der vorliegenden Arbeit, einem Projekt, das vom Österreichischen Wissenschaftsfonds (FWF, Projekt Nummer P21162) unterstützt wird, war, die physiologische Bedeutung von unkonjugiertem Bilirubin am Menschen zu untersuchen. Besonderes Augenmerk lag dabei auf der antioxidativen und antikanzerogenen Wirkung von Bilirubin.

Zu diesem Zweck wurden Personen mit leicht erhöhtem unkonjugierten Serumbilirubin, diagnostiziert mit Gilbert's Syndrom, und gesunde Probanden als Kontrollgruppe, rekrutiert. Insgesamt beendeten 100 Personen die Studie und gaben Blutproben ab. Die Serumkonzentration von unkonjugiertem Bilirubin wurde mittels HPLC bestimmt. Diese Konzentration war die Grundlage für die Zuordnung der Probanden in unterschiedliche Gruppen. Um die DNA Schäden zu messen, wurde die Methode des cytokinesis-block micronucleus assay, einer Standardmethode zur Messung von chromosomalen DNA Schäden am Menschen, angewandt. Dabei wurden Lymphozyten aus dem Blut isoliert, behandelt, auf Objektträgern aufgetragen und fixiert. Diese Zellen wurden unter dem Mikroskop untersucht und gezählt. In Zweikernzellen können Mikrokerne, nukleoplasmatische Brücken und nukleare Buds auftreten, welche auf DNA Schäden hinweisen und Biomarker für diese sind.

Es wurde gezeigt, dass Bilirubin antioxidative Wirkungen hat. Deshalb wurde die Hypothese aufgestellt, dass Personen mit leicht erhöhtem Bilirubinspiegel geringere DNA Schäden aufweisen, als die Kontrollgruppe. Es konnten jedoch keine signifikanten Unterschiede gezeigt werden, die Hypothese konnte also nicht bestätigt werden. Es wurde jedoch festgestellt, dass Mikrokerne mit dem Alter ansteigen und dass Frauen höhere Mikrokernwerte aufweisen als Männer. Dies könnte bedeuten, dass die Studienpopulation zu jung war (Durchschnittsalter: 32 Jahre) um signifikante Unterschiede zeigen zu können. Eine weitere Beobachtung war, dass Personen mit hohen Vitamin B12 Konzentrationen weniger DNA Schäden aufwiesen. Dies ist ein Hinweis auf den Einfluss dieses Vitamins auf den DNA Metabolismus.

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David Darling: http://www.daviddarling.info/encyclopedia/C/cell_cycle.html, (accessed on 08.06.2011).

CURRICULUM VITAE

PERSÖNLICHE DATEN

Name:	Katharina Marisch
Geburtsdatum:	17.10.1984
Staatsangehörigkeit:	Österreich

AUSBILDUNG

10/2003 – 02/2010	Studium Publizistik- und Kommunikations-wissenschaft an der Universität Wien
10/2004 – 07/2011	Studium Ernährungswissenschaften an der Universität Wien
09/1999 – 06/2003	Bundesoberstufenrealgymnasium Mistelbach, Niederösterreich
09/1995 – 06/1999	Hauptschule I Poysdorf, Niederösterreich
09/1991 – 06/1995	Volksschule Herrnbaumgarten, Niederösterreich

BERUFSERFAHRUNGEN und PRAKTIKA

09/2010	Institut für Ernährungswissenschaften, Universität Wien; Praktikum
08/2008 – 09/2009	Forum Ernährung heute, Wien; Praktikum
11/2008 – 03/2009	so what – Institut für Menschen mit Essstörungen, Wien; Praktikum
08/2006 – 03/2010	Niederösterreichische Nachrichten (NÖN), Redaktion Mistelbach; freie Mitarbeit