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Simone Maria Blassnigg

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Simone Maria Blassnigg

In Liebe und Dankbarkeit für

Mama und Papa

Christian

Meine beiden Omas

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IV List of Abbreviations

AD	Alzheimer´s disease
AKH	General Hospital of Vienna
BE	Broken egg
BIRNH	Belgium Interuniversity Research on Nutrition and Health
BMcyt	Buccal Micronucleus Cytome Assay
BMI	Body Mass Index
BNC	Binucleated cell
BR	Bilirubin
BV	Biliverdin
°C	Degree celcius
CAT	*Catalase*
CBMNcyt	Cytokinesis-block Micronucleus Assay
CC	Condensed chromatin
CO	Carbon monoxide
COPD	Chronic obstructive coronary disease
CNS	Crigler-Najjar Syndrome
CVD	Cardiovascular disease
DJS	Dubin-Johnson Syndrome
DNA	Deoxyribonucleic acid
% FMD	Flow mediated dilatation
FRAP	Fluorescence Recovery after Photobleaching
g	Gravitation
GPx	*Glutathione- peroxidase*
GS	Gilbert´s Syndrome
HbA1c	Hemoglobin A1c
HCl	Chloric acid
HDL	High density lipoprotein
HO-1/2/3	*Hemeoxigenase-1/2/3*
HPLC	High performance liquid chromatography
HSA	Human serum albumin
$HUMN_{XL}$	Human Micronucleus project on exfoliated buccal cells

IHD	Ischemic heart disease
kcal	Kilocalory
kg	Kilogram
KL	Karyolysis
KR	Karyorrhexis
LDL	Low density lipoprotein
LOOH	Linoleic acid hydroperoxide
LSC	Laser scanning cytometry
µL	Mycrolitre
M	Molar
MDA	Malondialdehyde
mg	Milligram
MN	Micronucleus
MNC	Cells containing micronuclei
NADPH oxidase	*Nicotinamide-adenine-dinucleotide-phosphate-oxidase*
NBUD	Nuclear bud
ND	Normal distribution
NHANES III	Third National Health and Nutrition Examination Survey
OATP	Organic anion transporter
P	Pyknosis
ROS	Reactive oxygen species
RS	Rotor Syndrome
SD	Standard deviation
SOD	*Superoxide dismutase*
TBOOH	t-butyl-hydroperoxide
TEAC	Trolox-equivalent antioxidant assay
THIN	The Health Improvement Network
UCB	Unconjugated bilirubin
UGT1A1	*Uridinediphosphate-glucuronosyltransferase*
wt/vol	weight/volume

1 INTRODUCTION

The present thesis was 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” and funded by the FWF-Austrian Science Fund. The project was performed at the Department of Nutritional Sciences of the University of Vienna and was furthermore supported by a scientific team of the Medical University of Vienna.

Bile pigments are coloured tetrapyrrols that arise in the liver during heme catabolism. For a long time especially its main components bilirubin and biliverdin were thought to have useless or even toxic properties. However, in particular the unconjugated form of bilirubin had come to researchers focus since antioxidative potentials have been reported. Therefore, a lot of *in vitro* and *in vivo* studies have been conducted until now aiming to investigate these actions. A certain condition in humans with mildly elevated unconjugated bilirubin levels is the Gilbert´s Syndrome. While strongly elevated levels of bilirubin may lead to severe neurologic damages, conditions seen in Gilbert´s Syndrome have been suggested to be even protective.

Oxidative stress may cause increased chromosomal and DNA damage which is known as a risk factor for developing cardiovascular disease and cancer. The Buccal Micronucleus Cytome Assay exhibits an optimal method for investigating chromosomal aberrations like micronuclei, broken eggs or binucleated cells. Not fully acknowledged nowadays this minimally invasive method may be predictive of risk factors like cancer or exposure to environmental substances on health status.

This study tried to combine these two fields and aimed to investigate the antioxidative properties of bilirubin in humans and their chromosomal protective effects. The authors’ interest focused on whether unconjugated bilirubin was able to prevent from chromosomal and DNA damage.

Hence, subjects with Gilbert´s Syndrome and matched healthy controls were recruited and their bilirubin concentrations as well as their chromosomal status were investigated.

The authors' hypothesis was that individuals with Gilbert´s Syndrome show less chromosomal damage than controls.

2 LITERATURE REVIEW

2.1 BILIRUBIN

Bilirubin, a yellow coloured molecule sensitive to oxidation and light, is the ultimate breakdown product of hemoglobin. This tetrapyrrole, a principle human bile pigment, was thought for a long time to have no physiological function than being a waste product of heme catabolism – useless at its best and toxic at its worst. Indeed, high concentrations of serum bilirubin may cause irreversible damages to the central nervous system, especially in neonates. Nevertheless, research of the past 20 years revealed a strong evidence for antioxidant and anti-inflammatory potential of bilirubin when blood levels are moderately elevated [Fevery, 2008, Vitek and Schwertner, 2007]. The following chapter focuses on bilirubin - its chemistry, metabolism, adverse and beneficial effects.

2.1.1 Bilirubin chemistry

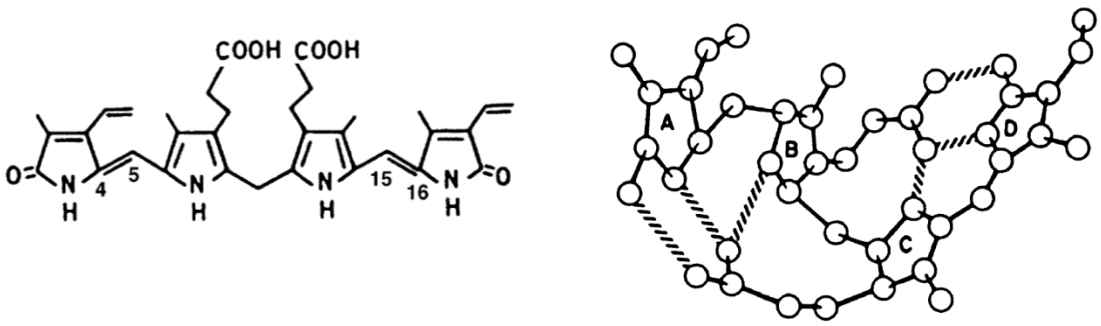


Figure 1: Chemical structure of bilirubin [Fevery, 2008].

Bile pigments, such as biliverdin (BV) and bilirubin (BR) are produced at a rate of about 300 mg per day in human adults as a result of heme breakdown [Zunszain et al., 2008]. The natural occurring form of unconjugated bilirubin (UCB) is the UCB molecule IXα 4Z, 15Z which is characterized by four pyrrole rings that are connected by carbon bridges (Figure 1). Two other isoforms IIIα

and XIIIα exist, which are formed by non-enzymatic processes by splitting UCB IXα into two halves. The nearly symmetrical and lipophilic UCB molecule is a di-carboxylic acid containing several polar functional groups [Vitek and Ostrow, 2009].

Determination of the crystal structure and the so-called “ridge-tile”- conformation brought new information. The interplanar angle between the dipyrrinone groups is about 100°. It allows each of the two acid groups to make three hydrogen bonds with the opposite dipyrrinone ring system and is responsible for its poor aqueous solubility. Because of its apolar properties bilirubin is strongly associated with specific proteins for further transportation in body fluids, such as albumin in plasma [Fevery, 2008, Zunszain et al., 2008, Ostrow et al., 1994].

The unstable UCB molecule is characterized by quickly oxidized double bonds and its high sensitivity to light. Furthermore, bilirubin reacts at low and high pH values [Vitek and Ostrow, 2009].

2.1.2 Bilirubin metabolism

Bilirubin is the result of hemoglobin catabolism in mammals. Hemoglobin is released by the breakdown of senescent red blood cells and in this metabolic process it is metabolised into free heme and globin. The heme pathway is summarized in Figure 2. Heme is reduced to biliverdin IXα by the rate limiting enzyme *hemeoxygenase-1(HO-1)* and one molecule of iron and CO is released. Heme can be toxic due to pro-oxidative effects and needs to be eliminated as fast as possible from the human body. In addition to HOs important role in heme degradation further facts about its possible preventive role were explored [Kim et al., 2011].

Until now, three isoforms of *HO (HO-1; HO-2; HO-3)* have been identified. *HO-1* is inducible; a lot of components like metals, cytokines, endotoxins, oxidants and vaso-active components as well as circumstances like heat shock or other forms of intra- or extracellular stress may lead to the enzymes activation. *HO-2*

on the contrary is constitutively expressed. It is mainly located at higher levels in the brain and the testes. *HO-3* is not expressed in humans and does not have a high catalytic activity. Studies show that especially *HO-1* has protective effects on ischemic diseases, hypertension, atherosclerosis and even diabetes mellitus [Kim et al., 2011].

In a second step biliverdin IXα is degraded to biliverdin IXα by *biliverdin reductase*. Bilirubins´ formation takes place in the monocytic macrophages of the spleen, in the bone marrow and in hepatic Kupffer cells [Sassa, 2006, Vitek and Schwertner, 2007, Fevery, 2008].

Every day about 4 mg heme/ kg bodyweight is produced. This amount equates to 250-300 mg bilirubin. In neonates more is formed. About 75-80 % of bilirubin arises from hemoglobin sources. Further 20-25 % are produced by non-hemoglobin heme-sources like myoglobin, cytochrome P 450 isoenzymes, *catalase, peroxidase* and *tryptophan pyrrolase*, that are primarily located in the liver. Conjugated (direct) and unconjugated (indirect) bilirubin (UCB) can be found in serum with UCB as the predominant one in healthy human subjects with an amount of 96 % [Wang et al., 2006, Vitek and Schwertner, 2007, Fevery, 2008].

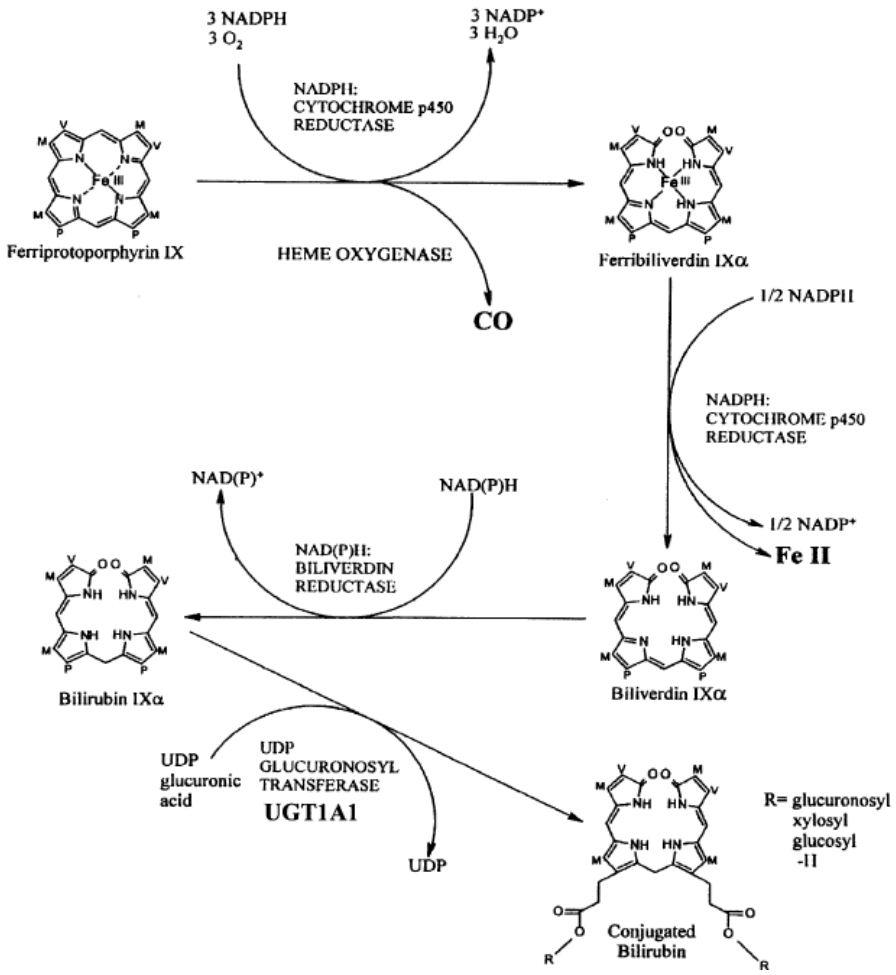


Figure 2 Heme pathway [Bosma, 2003].

For further transportation in the blood stream the non-polar UCB binds to human serum albumin (HSA) with high affinity. The albumin complex is transported to the liver where a passive uptake is guaranteed and also an active organic anion transporter (OATP) is known [Vitek and Ostrow, 2009, Toshinori et al., 2000].

Once reached hepatocytes bilirubin undergoes glucuronidation by conjugation with glucuronic acid, glucose or xylose. The lipophilic UCB molecule becomes hydrophilic which is important for being excreted into the bile. A crucial role for forming bilirubin glucuronides plays the enzyme *UDP-glucuronosyl-transferase -1A1* (*UGT1A1*) [Fevery, 2008, Shigeki et al., 2002].

In the intestinal lumen, conjugated bilirubin is promptly converted to urobilinogen and urobilins with support from *β-glucuronidases* and anaerobic bacteria. The molecules are reabsorbed by the intestinal mucosa, undergo enterohepatic circulation and a minor part is excreted by the kidneys. Urobilin is finally excreted via feces and contributes to its normal colour [Wang et al., 2006, Shigeki et al., 2002, Vitek and Ostrow, 2009].

2.1.3 Disturbed metabolism

Highly elevated serum bilirubin concentrations (UCB >20 mg/dL) may cause severe conditions in adults and neonates due to its toxicity. Normal concentrations of serum bilirubin add up to a maximum of 17.1 µmol/L (1 mg/dL). If total serum bilirubin concentrations exceed a level of 513 µmol/L (>30 mg/dL) the risk of developing neurological dysfunctions due to bilirubins brain toxicity is given. Bilirubin may trespass tissues and causes yellow colour in sclera and skin which is known as jaundice (*icterus*). In very severe forms UCB may also reach the blood brain barrier and if untreated may cause *kernicterus*. Beside the toxic form of hyperbilirubinemia a benign form called Gilbert´s Syndrome is known, which gained strong interest in the past decades [Silbernagl and Lang, 2005, Burke et al., 2009].

Pathologic forms of hyperbilirubinemia

Beside its known antioxidative function bilirubin can also be toxic at high concentrations that exceed the normal physiological ranges (>20 mg/dL). Higher bilirubin levels may be indicative of certain liver diseases or inherited dysfunction of bilirubin excretion and physiological outcomes depend strongly on its concentrations [Vitek and Schwertner, 2007, Bhutani et al., 2004, Wang et al., 2006].

The *prehepatic icterus* arises when bilirubin production is increased due to certain factors like hemolysis, inefficient reproduction of red blood cells or massive

blood transfusion. Drug and alcohol abuse as well as poisoning and hepatitis may cause damages of the liver. Defects of this organ cause *intrahepatic icterus* which goes along with the lack of excreting normal amounts of bilirubin. A *posthepatic icterus* is the result of a blocked bile duct, due to gall stones or tumours [Löffler, 2008, Silbernagl and Lang, 2005].

A *physiological icterus* can be observed in newborns as a consequence of hyperbilirubinemia. This icterus occurs when bilirubin production is elevated based on the increased degradation of fetal hemoglobin. A *pathological icterus* is developing if this hemolysis increases. Without any treatment elevated bilirubin levels lead to damages in certain areas of the brain, also known as *kernicterus.* Treatment can be assured through phototherapy. Irradiation of the newborns at wavelengths of 400-500 nm (e.g. overnight) is required in order to break down UCB. A second possible treatment is replacement-transfusion [Löffler, 2008, Silbernagl and Lang, 2005].

Crigler-Najjar-Syndrome (CNS)

CNS is found by a severe malfunction of *UDP-glucuronosyltransferase* due to a mutation of the UGT1A1 gene. The enzyme is completely (or nearly completely) absent and elimination of bilirubin is almost impossible and therefore leads, when untreated, to fatal symptoms. Two types can be distinguished. *Type I* is autosomal-recessive. Bilirubin levels are very high (>200 mg/dL) due to an absence of *glucuronosyltransferase*. Most of the children die of *kernicterus* before completing their first year of life if no liver transplantation was conducted and phototherapy failed. Only few cases are known worldwide. *Type II* is autosomally-dominant, the enzyme activity is reduced to <30 % of healthy subjects with plasma levels between 50 and 200 mg/L. Therapy is possible by inducing the enzyme with Phenobarbital [Strassburg, 2010b, Mutschler et al., 2007].

Benign hyperbilirubinemias

Dubin-Johnson-Syndrome and Rotor Syndrome (DJS/RS)

Both very rare disorders are inherited in an autosomal recessive mode. They have an elevation of unconjugated as well as conjugated bilirubin in common. Standard liver parameters are at normal range but DJS is characterized by a black coloured liver as a consequence of the presence of dark lysosomal melanin-like pigments. In Rotor latter cannot be obeyed. However, diagnosis through liver biopsy is not recommended. Distinction is warranted by analysis of the urine coproporphyrin-I excretion, which is found by 80 % in individuals with DJS slightly higher than in subjects with RS (65 %) and therefore serves as a differentiation criterion [Strassburg, 2010b].

Gilbert´s Syndrome (GS)

Gilbert´s Syndrome was first described by Gilbert and Lereboulet in 1901 and later by Meulengracht and Arias. The syndrome is characterized by mild, chronic, nonhemolytic unconjugated hyperbilirubinemia (UCB >17 µmol/L) and does not lead to liver inflammation, histological changes or progressive fibrosis [Yesilova et al., 2008, Strassburg, 2010a].

GS has a prevalence of 5-10 % of the Caucasian population and in general GS can be more often found in men than in women (12,4 % and 4,8 %) [Gilbert and Lereboulet, 1901, Radu and Atsmon, 2001, Bosma, 2003, Strassburg, 2010b, Strassburg, 2010a].

Elevated bilirubin levels in people with GS have a 30 % reduction of the normal hepatic activity of the enzyme *uridine-diphosphate-glucuronosyltransferase (UGT1A1).* A mutation of the promoter region which is called UGT1A1*28 is described and leads to additional TAs in the TATAA box due to a 20 % lowered transcription [Bosma, 2003].

Because of Gilbert Syndromes harmlessness a correct interpretation of the clinical presentation is necessary. Indeed, a higher risk is given when unwarranted invasive diagnostic methods, such as biopsies or endoscopic cholangiography, are used. When rightly interpreted, they should be avoided. In literature conditions as in GS are reported to be protective against cardiovascular disease and cancer due to antioxidant actions [Strassburg, 2010a].

2.1.4 Bilirubin – a potent antioxidant?

For a long time bilirubin was thought only to be a waste product in the human metabolism. Decades ago bilirubin was found to have strong antioxidative capacities. Besides reducing reactive oxygen species (ROS), and therefore oxidative damage, the bile pigment furthermore showed anti-mutagenic, anti-complementary, anti-inflammatory and anti-viral qualities [Bulmer et al., 2008b].

ROS generated by superoxide anion (O_2), hydroxyl radical (HO°) and hydrogen peroxide (H_2O_2) are inter alia contributing to the pathology of cancer, tumour promotion, aging, cardiovascular events and chronic inflammation. Prevention from oxidation is warranted through endogenous antioxidative enzymes like *superoxide dismutase*, *catalase* and *glutathione peroxidase* and exogenous antioxidants like Vitamins E, C and β-carotene [Stocker, 2004, Stocker et al., 1987b].

Bilirubin also plays a main role in oxidant defence due to H-donation to radicals. Therefore, the bile pigment is known for preventing oxidation of fatty acids, scavenging singlet oxygen, reducing peroxyl radicals and protecting human LDL from oxidation. Moreover, studies showed a synergistic effect of bile pigments with α-tocopherol in preventing membrane lipid peroxidation because it reduces the α-tocopherol radical. [Stocker, 2004, Stocker et al., 1987b].

It is demonstrated that UCB is >20 times more effective than Trolox (a Vitamin E analogue) on preventing *in vitro* LDL oxidation after Cu^{2+} addition. Under de-

fined conditions 17 µmol/L of UCB inhibits LDL oxidation whereas 500 µM Trolox are needed to receive the same results [Wu et al., 1994].

A former *in vitro* experiment by Stocker et al., 1987 aimed to measure antioxidant activity of bilirubin when linoleic acid is oxidised to linoleic acid hydroperoxide (LOOH) by a free radical chain mechanism. A significantly minor formation of LOOH could be observed in presence of low doses of bilirubin and its precursor biliverdin. Moreover a partial inhibition of the chain reaction was achieved through bilirubin. Therefore, the bile pigments are assumed to be able of scavenging peroxyl radicals [Stocker et al., 1987a].

Stocker et al., 2004 also mentioned that it is not clear to what extent bile pigments deploy anti-oxidant activity *in vivo*. A study by Datla et al., 2007 examined effects of *HO-1* induction on *NADPH-oxidase* activity in $ApoE^{0}$ mice and authors discovered that *HO-1* and bilirubin suppress the enzymes activity which reduces oxidative stress.

Furthermore Sedlak et al., 2009 showed an increased lipid oxidation in *HO-2* knockout-mice which goes along with a higher risk for neurotoxic damage and stroke because of reduced bilirubin levels [Sedlak et al., 2009]

Because of bilirubins´ antioxidative properties Mc Carty, M., 2007 hypothesizes that vascular as well as cancer risk may be reduced by donating *HO-1* inducers, bilirubin or biliverdin supplements or drugs that are able to decrease hepatic bilirubin conjugation (e.g.: Probenecid). The efficacy of Probenecid which is known as well-tolerated and the use of phycobilins originating from plants, algae and cyanobacteria may decrease *UGT1A1* and elevate UCB levels. Phycobilins show comparable antioxidative qualities as bilirubin and may therefore be interesting for being used for therapeutically measures [Mc Carty, 2007].

Bilirubin and cardiovascular disease

The antioxidative properties of UCB are assumed to reduce the risk for CVD. Vitek et al., 2002 investigated whether elevated bilirubin levels have positive effects on ischemic heart disease (IHD). Results showed a significantly lower prevalence of IHD in GS subjects (2 %) compared to the general population (12.1 %). Furthermore the total antioxidative capacity as well as HDL cholesterol was higher in GS subjects than controls. A protective role against developing IHD resulting from mild chronic hyperbilirubinemia (>17 µmol/L UCB) is assumed [Vitek et al., 2002].

Reduced risk for CVD has also been noticed in individuals with GS by Bulmer et al., 2008. Blood samples from subjects with GS and controls were determined for antioxidant status measuring. FRAP, TEAC, MDA, SOD, GPx, CAT and Cu^{2+} induced serum oxidation was evaluated. An increased lag phase of serum oxidation ($p= 0.020$) in comparison to the controls was assessed, showing a higher antioxidative capacity. Moreover, a trend for an elevated HDL: LDL ratio was found in the GS group ($p= 0.072$). These biomarker based results suggest a decreased prevalence for CVD in individuals with GS [Bulmer et al., 2008a].

A recent study by Yoshino et al., 2011 aimed to investigate the influence of serum bilirubin concentration on coronary endothelial function in overweight subjects. 107 patients without coronary artery disease were divided into an overweight (BMI $\geq$25 kg/m^2) and a normal weight group (BMI <25 kg/m^2). Beside estimation of serum total bilirubin, direct bilirubin, LDL-C, HDL-C and triglycerides were measured. The coronary vasoreactivity was measured by flow-mediated dilatation (% FMD). In the overweight group total bilirubin was significantly associated with % FMD ($p <0.05$) and HDL-C ($p <0.05$). These correlations could not be found in normal weight subjects and leads to the assumption that elevated bilirubin levels support endothelial function in overweight patients [Yoshino et al., 2011].

Bilirubin and cancer

In vivo studies

The association between serum bilirubin and cardiovascular and cancer mortality was investigated by Temme, et al., 2001. Data from 5460 men and 4843 women aged between 25 and 74 years were collected and analyzed in the Belgium Inter-university Research on Nutrition and Health (BIRNH) study. A baseline survey focusing on the relationship between diet and mortality was firstly conducted between 1981 and 1984. Ten years after the first data collection each subject was ascertained for vital status and cause of death. Average serum bilirubin levels were higher in men (0.44 mg/dL) compared to women (0.35 mg/dL). Results showed that men with higher serum bilirubin levels had a 58 % reduced risk of cancer mortality. The same trend was seen in women, who had a 24 % reduced risk, but no statistic significance could be established [Temme et al., 2001].

An epidemiological study conducted by Zucker et al., 2004 aimed to determine serum bilirubin levels of 16,865 subjects representing the U.S. population. Therefore, data of the Third National Health and Nutrition Examination Survey (NHANES III) collected between 1988 and 1994 were analysed. Men showed significantly higher UCB concentrations than women ($p < 0.001$). Results showed a significant inverse relationship between serum bilirubin concentrations and the incidence of colorectal cancer. A noticeable decrease in prevalence of colorectal cancer can be associated with an increase of each 1 mg/dL in serum bilirubin. Even though no classification in direct or indirect fractions was done the authors presume that preventive effects are resulting from the latter. Prevention from gastrointestinal malignancy by bilirubin can be assumed [Zucker et al., 2004].

Horsfall et al., 2011 examined the association between serum bilirubin levels and chronic obstructive coronary disease (COPD), lung cancer and all-cause mortality in a cohort of 504.206 subjects. Information was provided by The Health Improvement Network (THIN), a database from the United Kingdom that

includes more than 7 million people. Similar to the previously mentioned studies men had higher serum bilirubin levels (0.64 mg/dL) than women (0.53 mg/dL). Higher serum bilirubin concentrations were both in men and women associated with a decreased risk of lung cancer and all-cause mortality [Horsfall et al., 2011].

In vitro studies

Another study by Rao et al., 2006 investigated bilirubins´ action in human carcinoma cell lines. The results interestingly showed an antioxidative effect in hepatocytes- as well as pro-oxidative nature in human gastric carcinoma cell lines (TMK-1). An increase of ROS and DNA damage leads to coding for yet unknown apoptotic structures. TMK-1´s growth gets inhibited to nearly 50 %. Even if antioxidative effects of bilirubin are more frequent the authors assign anticancer effects due to pro-oxidative actions [Rao et al., 2006].

Although, there are plenty of studies that postulate the protective effects of UCB *in vivo* a recent hypothesis by Astolfi, et al., 2011 links decreased UGT1A1 action in patients having GS with a higher risk for breast cancer. Inactivation of potentially carcinogenic 4-OH estrogens through the enzyme is lowered; accumulation may lead to a higher risk for developing tumours in breast tissue. Authors rely on two studies associating *UGT1A1* with a 28 time higher risk for breast cancer. However, data evaluating GS and breast cancer risk are rare and further investigations *in vitro* and *in vivo* are necessary for proving this theory [Astolfi et al., 2011].

The relationship between bile pigments and its anti-mutagenic actions was investigated by Bulmer et al., 2007 performing the Ames *Salmonella* test in three bacterial strains (TA98, TA100, and TA102). Results admit anti-mutagenic potential to bilirubin and biliverdin in presence of several mutagens (e.g.: TBOOH). Arimoto, et al., 1980 who able to show protective effects of hemin, bilirubin and biliverdin on mutagenic influences of benzo[α]pyrene [Bulmer et al., 2007].

Bilirubin may be seen as a biomarker for CVD and related diseases. So far, the role of BR as a biomarker for cancer has not been fully acknowledged although epidemiological studies clearly indicated a cancer protective role for BR [Lin et al., 2010, Breimer and Mikhailidis, 2011].

2.2 DNA DAMAGE

DNA underlies permanent hazards because of environmental or cellular processes. Especially oxidative stress challenges the cells defence and often its implications lead to cancer, neurodegenerative diseases and cardiovascular risk. The following chapter highlights factors leading to DNA damage and the most popular assessment methods. Moreover, it focuses in particular on the potential of "The Micronucleus Cytome Assay" as a tool for monitoring chromosomal and DNA defects in different tissues [Jackson and Bartek, 2009, Thomas et al., 2009].

2.2.1 DNA damage and ROS production

The DNA, carrier of the human genetic code can be damaged throughout a wide range of reasons. Each of the ~10^{13} cells in the human body receives thousands of DNA lesions every day. Impaired or incorrect repair due to blocked genome replication and transcription may lead to mutations or genome aberrations causing an increased risk for diverse cancers, neurodegenerative or cardiovascular diseases. DNA damage arises also by other mechanisms due to oxidative stress. Cellular processes, external factors and/or disease states can lead to the formation of reactive oxygen species (ROS) that may interact with the DNA. On the one hand ROS are released from phagocytes to destroy cells infected with virus or bacteria and on the other hand formed by ionizing and ultraviolet radiation or potent environmental carcinogens and chemicals in mitochondria (Figure 3). Their negative impacts are based on an overload of O_2^-, HO° or H_2O_2 and furthermore lead besides genomic alterations to enhanced lipid oxidation, protein oxidation and DNA oxidation [Cooke, 2003, Ferguson et al., 2006, Jackson and Bartek, 2009].

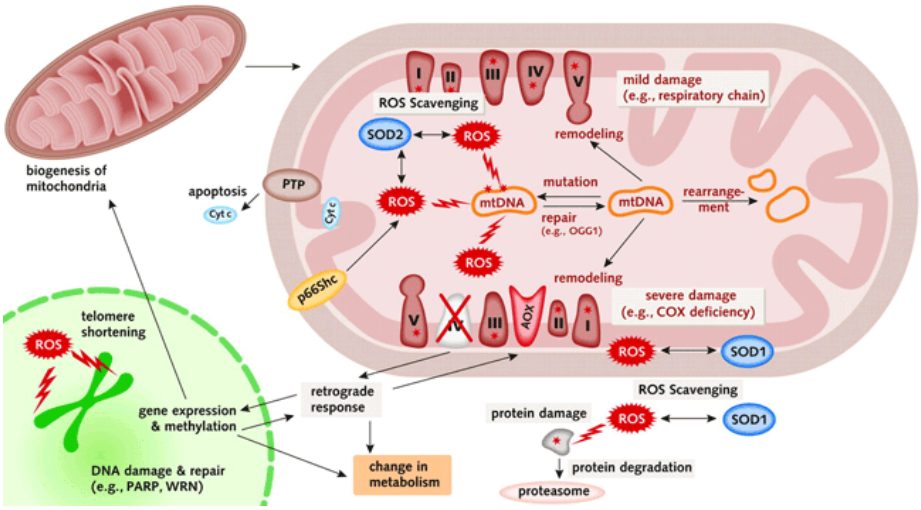


Figure 3 shows the impact of ROS on DNA and proteins. ROS are generated in mitochondria, scavenging systems exist and if mitochondrial DNA functions well damaged proteins get removed and replaced by new ones. In more severe cases as seen in Complex IV deficiency expression of additional genes gets stimulated to rescue lost functions. Oxidative damage furthermore leads to induction of apoptosis [Jansen-Dürr and Osiewazc, 2002].

2.2.2 Methods for estimation of DNA damage

Since oxidative stress and its consequences lead to an increased health risk it is necessary to measure the status of DNA damage in humans. Different biomarkers have been designed for a better mechanistic understanding on DNA and chromosomal level. One possibility is measuring DNA lesions such as 8-OxodG in urine or leukocytes, lymphocytes or mononuclear blood cells after influence of oxidative stress. These modified DNA bases represent one of the most common lesions that are indicative for failed DNA repair. More direct methods include measurements in peripheral blood lymphocytes and buccal cells. Single gel electrophoresis (COMET assay) provides investigation of DNA single or double-strand breaks and other oxidised DNA bases in lymphocytes and can be adapted (with concern to some limitations) also in buccal cells. In both, lymphocytes and buccal cells chromosomal aberrations as micronuclei can be obtained and related to chromosomal damage. This assay already accredited in lymphocytes shows also huge potential as well in buccal tissue for determination of chromosomal alterations and is described in the following section [Dhillon et al., 2004, Ferguson et al., 2006, Lee and Pfeifer, 2008].

2.2.3 Buccal Micronucleus Cytome Assay (BMcyt)

The Micronucleus (MN) assay in exfoliated buccal cells has already been used since the 1980s. It represents a minimally invasive and useful method for investigating DNA damage, chromosomal instability, cell death and the regenerative capacity of human buccal tissue. Especially in long-term studies impacts of lifestyle, nutrition or genotoxine exposure can be easily determined by harvesting cells via a toothbrush, subsequent preparation and analysis. Human buccal mucosa is a barrier for potential pathogenic or carcinogenic compounds, and indeed, about 90 % of human cancers have their origin in epithelial cells. Therefore, it seems self-evident that oral epithelial cells represent a perfect tool for biomonitoring risk of diverse diseases *in vivo* [Holland et al., 2008, Thomas et al., 2009].

By contrast to the cytokinesis-block micronucleus assay (CBMNcyt) which is applied in peripheral blood lymphocytes no standardized protocol for the BMcyt is available to date. For solving among other topics the issue of unification the "Human MicroNucleus project on eXfoLiated buccal cells" ($HUMN_{XL}$) was launched. The projects aim is to refine the assay by strong collaboration with international laboratories [Bonassi et al., 2011].

Furthermore first approaches for automation of the BMcyt are described by Leifert et al., 2011. Laser scanning cytometry (LSC) is suggested to measure biomarkers for DNA damage (micronuclei, broken eggs), cytokinesis defects (binucleated cells) or cell death (condensed chromatin, karyorrhexis, pyknosis and karyolitic cells) following standardized scoring criteria. Automation may contribute to maintaining uniform results [Leifert et al., 2011].

Compared to the CBMNcyt no blood samples need to be taken for the BMcyt and buccal tissue is easily reachable and therefore, the method serves perfectly for large biomonitoring studies, especially in pediatric populations. Easy storing of samples and low costs favour this method. Since buccal tissue allows immediate assessment of DNA damage no further replication step is required. The BMcyt is successful without establishing cell cultures for analysing meta- and in-

terphase in binucleated lymphocytes. The strong correlation of MN frequency in buccal cells with MN frequencies in lymphocytes warrants great potential in biomonitoring risks for certain diseases, in the first place for cancer [Holland et al., 2008, Bonassi et al., 2011].

2.2.4 The origin of chromosomal damage

Endpoints and other nuclear anomalies in the BMcyt

The oral epithelium is built up of four strata, the *lamina propria, stratum basale*, *stratum spinosum* and a keratinized layer at the surface. Micronuclei can be measured in erythrocytes, lymphocytes and exfoliated epithelial cells. They mainly descend from chromosome fragments or whole chromosomes which lag behind at anaphase during nuclear division. Such displaced chromosomes or fragments are eventually enclosed by a nuclear membrane and can be seen after staining a smaller round part next to the main nucleus [Holland et al., 2008, Fenech et al., 2010].

MN are firstly expressed in the basal layer, which contains stem cells that may undergo genetic changes. After a period of 7-10 days the cells migrate from the basal layer to the surface. Daughter cells which may have MN finally reach the surface and exfoliate into the buccal cavity. Seldom, some cells get blocked in a binucleated stage or form broken eggs (also known as nuclear buds). They represent biomarkers of defect cytokinesis and gene amplification, respectively. In addition to the mentioned ones biomarkers of apoptosis and necrosis (condensed chromatin, Karyorrhexis, Pyknosis and Karyolysis) can be observed (Figure 4); [Holland et al., 2008].

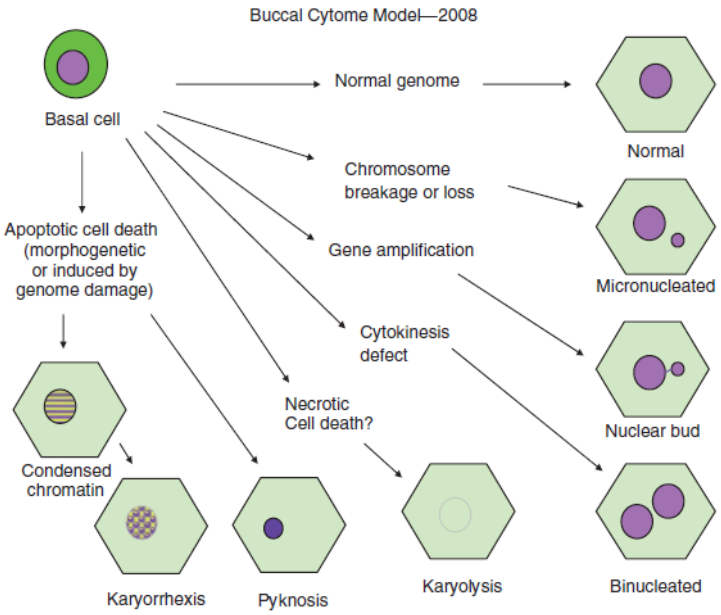


Figure 4: Various cell types that are scored in the BMcyt [Thomas et al., 2009].

2.2.5 BMcyt and diseases

Alzheimer

First ones to perform the BMcyt in patients suffering from Alzheimer´s disease (AD) were Thomas et al., 2007. Their aim was to establish whether MNs or other parameters reflecting genome damage or cell death could be used as a risk marker for the development of the disorder. Results did not show notable differences in MN and BE frequency between the AD and control groups. Significantly lower frequencies of basal cells, condensed chromatin and karyorrhectic cells (all $p < 0.0001$) in AD subjects where found which may be a sign for changes in cellular kinetics or structural profile of buccal mucosa. Authors suggest that a combination of basal and karyorrhectic cells as biomarkers along with measurement of AD-specific proteins (tau and β-amyloid) might play an important role for detecting individuals with increased risk of AD [Thomas et al., 2007b].

Down´s Syndrome

The BMcyt was used for investigating the impact of ageing in healthy young and old subjects and in a young Down´s Syndrome cohort on cell anomaly frequency. Normal ageing accompanied with decreased cell and DNA repair mechanisms and further results showed a significant increase of MN ($p < 0.05$, average increase +366 %), KR ($p < 0.001$, average increase +439 %), CC ($p < 0.01$, average increase +45.8 %), and basal cells ($p < 0.001$, average increase +233 %) compared to young controls. In Down´s Syndrome which is characterized by premature ageing an even more significant increase in MN, BN and a significant decrease in CC, KR, and P in contrast to the healthy young group was observed. Therefore, the BMcyt may be seen as an effective method for identifying changes of DNA and different cell types while normal and premature ageing [Thomas et al., 2007a].

Cancer

Dorea, et al., 2012 focused on the appearance of cytological abnormalities in patients with oral malignant neoplasms and healthy subjects using the BMcyt. Significantly higher occurrence of MN can be seen in cells collected from lesions than in cells from intact areas, independently from cancer pre-or absence ($p < 0.0001$). Furthermore MN were significantly more frequent in smokers and mouthwash users ($p < 0.0001$). Indicators for apoptosis were also measured (CC, KR; P), showing a lower frequency in cells from lesions than in cells from normal areas and control group. Apoptosis in buccal tissue might be impaired due to this kind of cancer. Investigation of cell alterations in the oral cavity using the BMcyt was proved as useful [Dórea et al., 2012].

In the study from Dey et al. buccal cells of patients with breast carcinoma and patients having benign breast lesions were collected. Micronucleus frequency in buccal mucosa was significantly higher in those suffering from carcinoma than in subjects with benign breast lesions ($p < 0.001$). Performing the BMcyt and especially scoring MN should therefore serve as an early detection method for high risk breast carcinoma cases [Dey et al., 2010].

2.2.6 BMcyt -Life style and exposure

The micronucleus frequency in hairdressers and controls was assessed by Rickes et al., 2010. Since this occupational group is exposed daily to harmful chemical substances, such as p-phenylenediamine, hydrogen peroxide or thioglycolic acid in colourings and straigtheners it was in the researchers' interest to determine their impact on DNA damage and chromosomal instability. Results clearly showed that all parameters (MN, BNC, BE, and the sum of all anomalies) are significantly higher in hairdressers ($p=0.0001$). A higher genotoxic load could be observed in hairdressers concluding that their products used are harmful for men [Rickes et al., 2010].

Yadav and Sharma 2008 used the BMcyt for assessing genotoxic effects of low frequency electromagnetic radiation emitted by cell phones on humans. For regular phone users the number of micronucleated cells was higher compared to non-users. A positive correlation between years of exposure and MN frequency was found even though a slight increase of MN could be observed in subjects whose exposure exceeds 4 years. A similar study conducted by Hintzsche and Stopper, investigated the same issue. In contrary their findings did not show increased micronuclei frequency in mobile phone users [Hintzsche and Stopper, 2010, Yadav and Sharma, 2008].

Drinking water contaminated with arsenic is a risk factor for developing different types of cancer. Bartolotta et al., 2011 therefore evaluated MN frequency in an exposed rural population and in an urban population in Argentina. The exposed group showed a significant increase in MN frequency compared to controls ($p= 0.0005$). Hence, determination of cell aberrations in buccal mucosa could be a promising method for measuring potential genetic risk through environmental contaminants [Bartolotta et al., 2011].

Effects of antioxidants on MN frequency have been reviewed by Thomas et al., 2010 for both lymphocytes and buccal cells. In the latter significant reductions of MN frequencies were obtained through supplementation of high doses of folate, α-tocopherol or β-carotene in different cohorts [Thomas et al., 2010].

Even though intervention with single micronutrients or their combinations have antioxidative and therefore beneficial effects on MN frequency it is necessary to investigate effects of endogenous antioxidants, such as bilirubin. Therefore, the present thesis investigated the effect of mildly elevated UCB levels on the BMcyt in order to investigate chromosomal modifications in subjects with GS.

3 MATERIALS AND METHODS

3.1 Study Design

The aim of the recent study was to determine whether the antigenotoxic effects of bilirubin prevent chromosomal damage in individuals with mildly elevated circulating serum bilirubin (Gilbert Syndrome). The study was conducted in a cross-sectional design and was performed over a period of two years.

Until now only little information about the effects of oxidative DNA damage in people with Gilbert Syndrome is available. Out of this reason the study aimed to investigate levels of DNA stability in GS and control subjects.

It was hypothesized that the cells of the buccal tissue from GS subjects would be protected from oxidative induced chromosomal damage due to elevated bilirubin levels.

For the screening a small amount of blood was taken from the subjects after the patients´ agreement was filled. A questionnaire checked whether the subjects met the inclusion criteria (see criteria below). In case the subjects met them, a larger blood sample was obtained at the general hospital of Vienna (AKH) and the participants were classified in GS and control.

Participants were advised to fast and only ingest 400 kcal for 24 hours, before the second, main blood collection. As a consequence of fasting, serum bilirubin levels rise, especially in people with GS [Owens and Sherlock, 1973]. The next morning, buccal cells, blood and urine samples were collected. Buccal cells were prepared on slides and stored in boxes at the Institute of Cancer Research in Vienna.

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 were not aware whether subjects had high or low serum bilirubin levels.

Study population

This study was approved by the Human Ethics Research Committee of the AKH (Ref. No. 274/2010). A patient agreement had to be signed by participants. All samples were collected from April to September 2010.

In total 100 subjects, aged between 20 and 80 years, were recruited for the study. The 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, serum liver enzyme activities and blood counts in the normal range were required in addition to increased bilirubin levels. 45 participants were diagnosed with GS and 39 were then allocated into the control group. For 16 subjects the assignment could not be performed due to unclear bilirubin levels around 1 mg/dL and were just included for statistical analysis using bilirubin tertiles. Participants were recruited partly by doctors in the AKH and furthermore, by advertisement posted in universities, pharmacies or hospitals, as well as on internet platforms and in newspapers.

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 (<1.2mg/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 and 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)

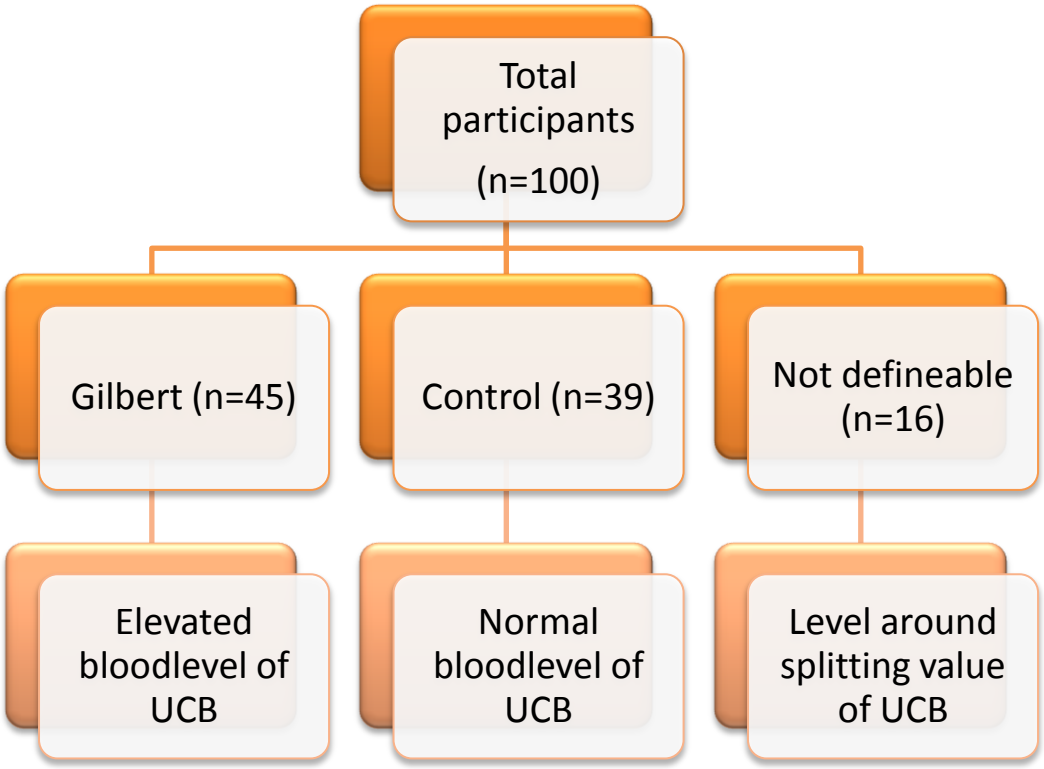


Figure 5: Distribution of the study population

3.2 BMcyt – Equipment

The accomplishment of this assay for the present study based on the protocol of Thomas et al; 2009.

Name	**Unit**	**Producer**
Tooth brush		Mentadent
Plastic tubes	50 mL	Sarstedt
Centrifuge cups	1,5 mL	Carl Roth GmbH+Co. KG
Abacteria Pipettes	10 mL	Chase
Slides and coverslips		HD Schientific
Coplin jars		Biolab

Table 1: Equipment used for the BMcyt

3.3 Chemicals

Name	**Unit**	**Producer**
Physiologic salt solution	0.9 %	Sigma- Aldrich
Ethanol		Merck
Bidistilled water		Institute of Cancer Research, MedUni, Vienna
Chloric acid	5 M	Merck
Schiff's Reagent		Sigma-Aldrich
Light Green	0,20%	Gurr's
DePex		Merck
Methanol	80%	Carl Roth GmbH+Co. KG

Table 2: Chemicals used for the BMcyt

3.4 Collection of buccal cells

The subjects were asked to rinse their mouths twice with water before the cell collection. This is an important step to remove excess debris. For each subject a topped container with 10 mL salt solution has been prepared before the collection. The container was provided with the subject's code and the date of collection.

Participants had to rotate a small-headed toothbrush 10 times against the inside of their cheek walls in a circular motion. They were advised not to push too much while using the toothbrush to avoid potential irritations and bleedings in this sensitive area which could impurify the sample.

The samples from the toothbrush were collected in the salt solution and the process was repeated twice on each cheek.

Subsequently the containers were closed and stored at 4°C until slides were prepared.

3.5 Harvesting buccal cells and slide preparation

The collected cells were put into TV-10 centrifuge tubes and centrifuged for 10 minutes at 581 g at room temperature.

The supernatant was removed; about 1 mL of the cell suspension was added with 5 mL salt solution.

A second centrifugation for 10 minutes at 581 g at room temperature followed. This procedure was repeated once more. After two washing steps in salt solution the best results were maintained. Using salt solution warrants inactivation of endogenous *DNAses* and removal of bacteria and cell debris.

Again the salt solution was added, cell suspension got homogenized for 2-3 minutes and then centrifuged under the known conditions (duration: 10 minutes, 581 g; room temperature)

3.6 Fixing buccal cells

After removing the supernatant the samples were displaced with 2-3 mL of 80 % methanol depending on the number of buccal cells. The samples were then stored for fixing at 20°C for 20 minutes.

120-150 µL of the cell suspension were dropped on precoded microscope slides and dried in the air for about 10 minutes before staining.

3.7 Staining for microscopy

After drying slides were put into Coplin jars containing 5 M HCl for 30 minutes. Then they were well rinsed in running tap water.

For testing the efficacy of the treatment with HCl a negative control slide was bathed in distilled water for 30 minutes instead of 5 M HCl.

Next the drained slides were placed into Coplin jars containing Schiff´s Reagent for 90 minutes in a dark environment at room temperature. Since there was no separate dark room available the jars were covered with aluminium foil.

After 90 minutes the reagent was removed. The slides were first washed in running tap water and afterwards washed in distilled water.

As a next step slides were immersed in Coplin jars containing 0.2 % (wt/vol) light Green. After 20-30 seconds slides were rinsed well in distilled water.

To blot away any residual moisture the slides were immediately placed vertically. Moreover it is important not to apply any pressure or rub on the cells as this may dislodge and damage the cells. For complete drying slides were placed under the laboratory hood over night.

3.8 Covering the slides

The completely dried slides were covered with coverslips under a laboratory hood. Using gloves is required because organic solvents in the glue (DePex) may provoke irritations of the respiratory tract and the skin. Some drops of DePex were placed on the slides by plastic pipette and immediately coated with a coverslip. Excess air bubbles were expelled by pressing the coverslip gently. Excess DePex got wiped away from the edges with some paper towel. Furthermore it had to be secured that DePex was neither on top of the coverslips nor on the coding area.

Finished slides were placed on a tray to be dried under the laboratory hood overnight. After drying slides were stored in special boxes at room temperature until evaluation.

3.9 Microscopy

Slides were scored for chromosomal damage biomarkers under the microscope. Nuclei and micronuclei are stained in magenta whereas the cytoplasm appears pale blue/green. In negative controls without 5 M HCl treatment the nuclei will not be stained with magenta colour.

3.10 Scoring criteria

Cells were scored under a transmitted light microscope using a magnification of 400. In some cases a magnification of 1000 and the use of immersion oil was necessary to ensure the best possible examination.

First the frequency of all cell types in a minimum of 1000 cells was counted and following the frequency of DNA damage biomarkers (MN and BE) was scored in a minimum of 2000 differentiated cells. The frequencies of the cell types in the assay were then calculated as the number of cells per 1000 differentiated cells.

3.11 Cell types

3.11.1 Differentiated cells

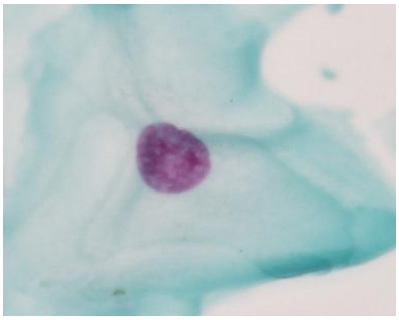
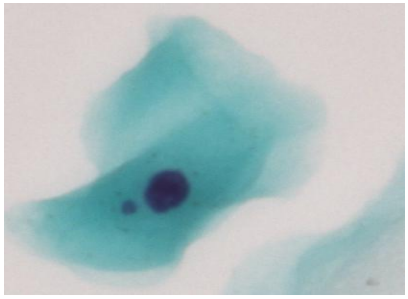


Figure 6: Differentiated cell

Differentiated cells are more angular and flatter than basal cells. Their nucleus is round and uniformly stained. This cell type has a smaller nuclear:cytoplasm ratio. Apart from the nucleus no other structures containing DNA can be found in differentiated cells [Thomas et al., 2009].

3.11.2 Cells containing Micronuclei (MN)



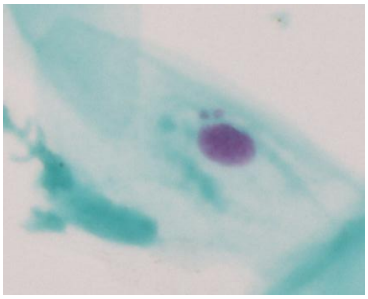


Figure 7: Cells with micronuclei

In both cell types, basal or differentiated, micronuclei can be obeyed. Out of practical reasons MN in basal cells are not getting counted due to the low frequency of this cell type. In cells with MN a main nucleus as well as one or more smaller structures called micronuclei can be seen. MN are characterized by a round or sometimes oval shape and their size is between 1/3 to 1/6 of that of the main nucleus. MN have the same staining intensity and texture as the main nucleus. Furthermore the MN must be located within the cytoplasm of the cells. MN are indicative for chromosomal damage or fragmentation during nuclear di-

vision. In buccal mucosa the frequencies for micronucleated cells are usually in a range of 0.5-2.5 MN per 1000 cells [Holland et al., 2008].

3.11.3 Broken Eggs (BE)

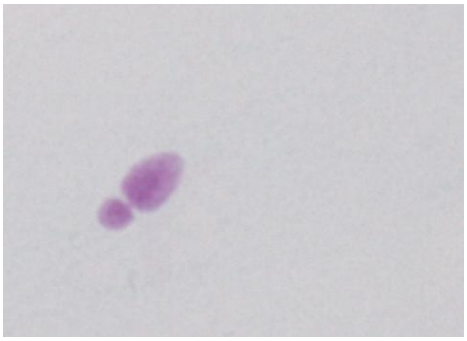


Figure 8: Broken Egg

BE are also called nuclear buds (NBuds) since nuclear material gets eliminated by budding [Tolbert et al., 1991]. The so called NBud is still attached to the main nucleus and has a similar staining intensity. BE reach a size of about ¼ to ½ of the main nucleus [Nersesyan, 2005].

3.11.4 Binucleated cells (BNC)

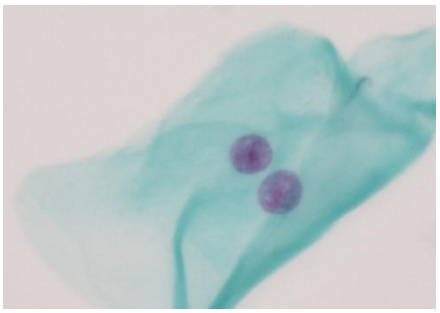


Figure 9: Binucleated cell

Binucleated cells contain two main nuclei of a similar size which are usually very close and may touch each other. BNC show the same morphology as seen in normal cells. Until now the significance of this cell type is not known but they are probably indicative of failed cytokinesis [Shi and King, 2005]. BNC may also be important biomarkers for identifying aneuploidy, which can be observed in people suffering from Down´s Syndrome [Thomas et al., 2007a, Thomas and Fenech, 2007].

3.11.5 Condensed Chromatin (CC)

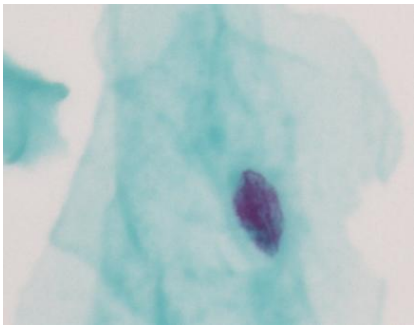


Figure 10: Condensed Chromatin

In buccal cells with condensed chromatin the nucleus shows areas of aggregated chromatin that is visible in striated nuclear patterns. Some areas are more intensively stained which is an apparent sign that chromatin aggregates in some regions while it gets lost in other areas [Wyllie, 1981]. It is assumed that these cells may indicate early stages of apoptosis, but until now this has not been proven [Thomas et al., 2009]

3.11.6 Karyorrhectic cells (KR)

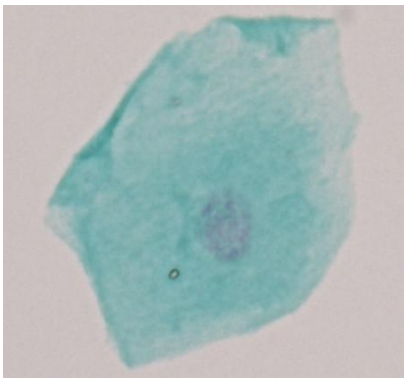


Figure 11: Karyorrhectic cell

In contrast to cells with condensed chromatin karyorrhectic cells are distinguished by a more extensive nuclear chromatin aggregation. They have a densely speckled nuclear pattern due to fragmentation in the late phase of apoptosis. So far this was not shown conclusively [Wyllie, 1981, Tolbert et al., 1991].

3.11.7 Pyknotic cells (P)

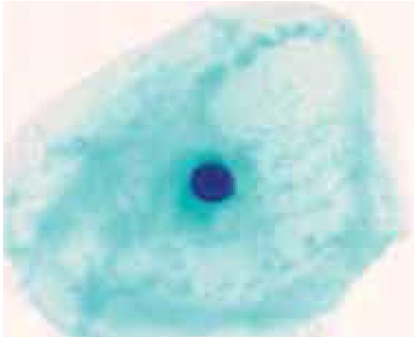


Figure 12: Pyknotic cell

This cell type is characterized by a small shrunken nucleus which is uniformly and very intensely stained [Wyllie, 1981, Tolbert et al., 1991]. Usually the nuclear diameter averages out 1/3 to 2/3 of the main nucleus´ normal size. Their biological significance and the mechanism leading to their formation are not known yet but it is assumed that pyknotic cells may be undergoing a unique form of apoptosis or necrosis. It is thought that they may represent an alternative mechanism of nuclear disintegration that is different from the process lead-

ing to condensed chromatin and karyorrhectic cell death stages [Holland et al., 2008, Chen, 2006].

3.11.8 Karyolytic cells (KL)

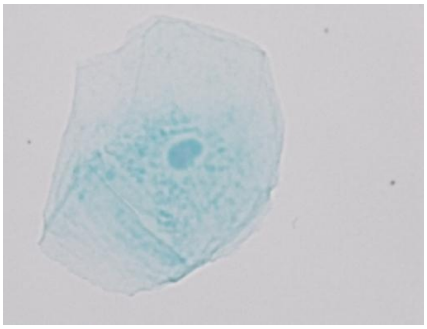


Figure 13: Karyolytic cell

This cell type represents a very late stage in the cell death process and may be indicative of necrosis. The main nucleus does not contain any structures of DNA. Due to depletion of DNA no staining via Feulgen is given. The cytoplasm as well as the nucleus appears in a slightly green colour. Therefore, the nucleus can hardly be seen and due to their ghost-like appearance they are also called “ghost cells” in literature [Wyllie, 1981, Tolbert et al., 1991].

Condensed chromatin and karyorrhectic cells were recorded in one category since even among scientist some uncertainties are discussed.

The pictures for this section were taken by the author and Alice Petschnig.

3.12 Statistical analysis

Determined data were statistically analyzed using SPSS Statistics 17.0.

All statistics referring to cell anomaly frequency in the study were reported as the number of anomaly per 1000 differentiated cells.

Normal distribution (ND) was tested in defined groups by using the Kolmogorov-Smirnov test.

For normal distributed data T-test was performed to evaluate differences of mean in 2 groups and Oneway ANOVA including a post hoc test was conducted for more than 2 groups.

If ND was not given Mann-Whitney-U-Test was performed in 2 groups and. Kruskill-Wallis-H-Test including ANOVA post hoc tests were accomplished in more than 2 groups.

For detecting correlations between 2 parameters Pearson coefficient (ND) or Spearman´s rho (not ND) was used.

A level of $p < 0.05$ was considered significant and marked with *.

4 RESULTS AND DISCUSSION

The aim of the present study was to investigate the relationship between bilirubin concentration and DNA damage. Furthermore, it was of high interest whether subjects with Gilbert´s syndrome and therefore mildly elevated bilirubin levels show less signs of DNA damage compared to control subjects.

100 individuals participated in the study; general criteria of the study population can be seen in Table 3. After the parameters for each individual have been counted, statistical analysis was carried out. Results are to be presented in this section.

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

Data are means ± SD.

Table 3: General description of the study population

4.1 DNA damage

Every individual was investigated with special focus on indications for DNA damage such as micronuclei and broken eggs using the BMcyt. Cells with micronuclei and numbers of micronuclei were reported, since one cell sometimes can obtain more than one micronucleus.

Mean DNA damage in the whole study population presented as the number of anomalies per 1000 differentiated cells was 0.27 ± 0.36 for cells with micronuclei, 0.32± 0.43 for the number of micronuclei and 0.60 ± 0.59 for broken eggs.

4.1.1 DNA damage in Gilbert´s Syndrome and Controls

Subjects were divided into the GS or control group according to the level of unconjugated bilirubin which was detected by HPLC (for further information see the diploma thesis by Katharina Marisch “The DNA protecting effect of unconjugated bilirubin”) [Marisch, 2011]. UCB levels were defined for GS group as >1 mg/dL (17.1 µmol/L and for the control group as <1 mg/dL (17.1 µmol/L). Results are shown in Table 4.

	Gilbert´s Syndrome	Controls	p-Value
Number	45	39	
MNC	0.25 ± 0.36	0.29 ± 0.38	0.607
MN	0.28 ± 0.41	0.34 ± 0.46	0.590
BE	0.53 ± 0.50	0.61 ± 0.63	0.619
BNC	11.50 ± 4.77	11.60 ± 4.39	0.918
CC/KR	9.85 ± 5.51	9.83 ± 5.18	0.986
KL	45.35 ± 55.01	31.43 ± 31.63	0.265
P	1.16 ± 0.87	1.17 ± 0.89	0.948

Data are means ± SD.

Table 4: DNA damage in GS and control

Between GS and control group no significant results were obtained. However, a 16 % lower mean in the number of cells with micronuclei as well as a 14 % lower mean in the number of micronuclei was seen in GS group compared to controls. Furthermore, the latter had a 31 % higher number of karyolytic cells than subjects with GS (Figure 14).

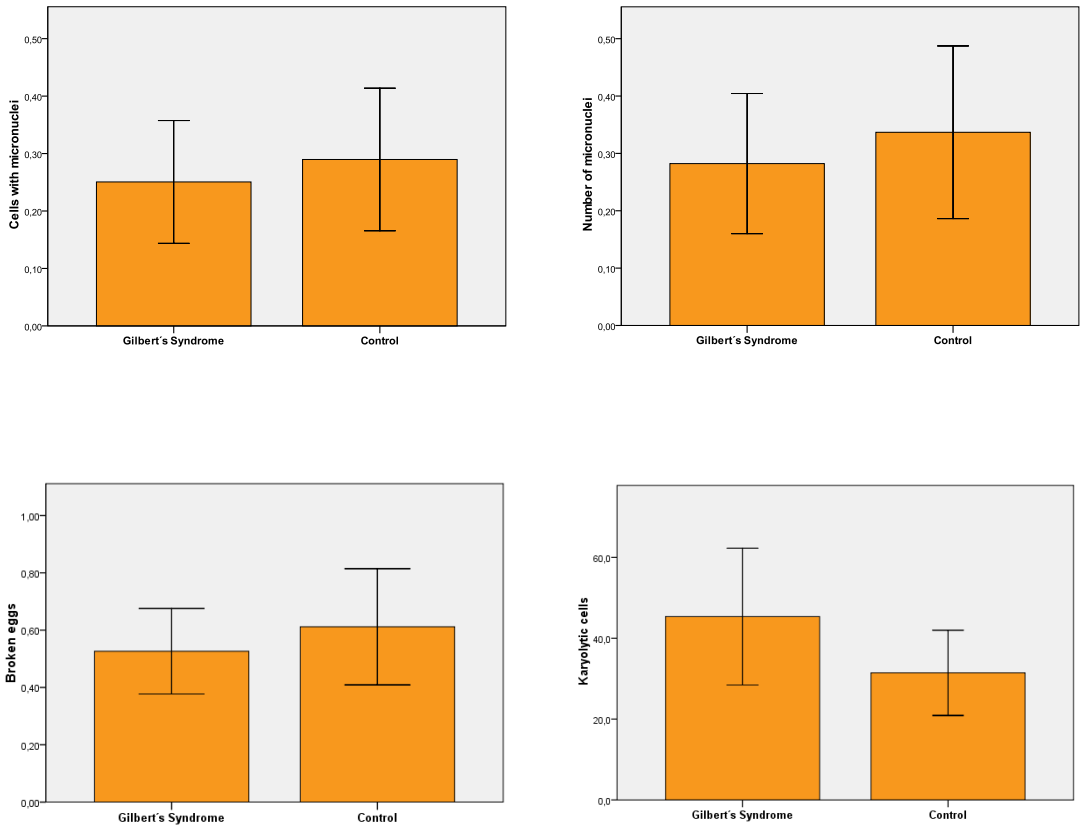


Figure 14: DNA damage in GS and control

Significantly lower levels of DNA damage were expected in subjects with Gilbert´s Syndrome due to elevated levels of unconjugated bilirubin and its therefore proposed antioxidative potential. However, insignificantly lower numbers (in %) of biomarkers for genotoxicity in GS were noted, which contributes to cardioprotective effects of UCB as reported in literature.

For the first time a study like this was conducted investigating the effects of increased bilirubin levels towards DNA damage by using the Buccal Micronucleus Cytome Assay. Therefore, a comparison of the obtained results with existing literature is not possible. Further research is needed to gain more information on possible effects of bilirubin on DNA stability.

The ranges of micronuclei were within 0.5 – 2.5 MN/ 1000 differentiated cells in both GS and control group which goes along with other reports [Thomas et al., 2009].

Thomas et al., 2009 furthermore report that among other factors smoking status and age contribute to higher levels of oxidative stress. Most of the participants in the study were relatively young and all were non-smokers (up to 5 cigarettes). A low level of oxidative stress was therefore assumed in both groups [Thomas et al., 2009].

What supports this assumption is that results from the thesis by Alice Petschnig “Einfluss einer Ernährungsintervention auf das Auftreten von Mikrokernen in Mundschleimhautzellen von Typ II-Diabetikern“ showed significant differences in the number of broken eggs between former smokers and non-smokers. Ex-smokers obtained higher numbers of this biomarker ($p= 0.02$). This may contribute to the sensitivity of the method and to the quality of the analysis [Petschnig, 2011].

Further results of the mentioned thesis showed that with increased HbA1c levels (≥7.01 %) the number of pyknotic cells rises as well. HbA1c is the main marker for evaluating long-term blood sugar regulation and levels exceeding 7 % are indicative of diabetes. A combination of already established biomarkers like HbA1c and biomarkers measured in the BMcyt could be supportive in estimating health risks.

A study by Dhillon et al., 2004 showed that buccal cells have a shorter turnover than lymphocytes and a less active DNA repair system what might indicate a higher sensitivity for detection of DNA damage [Dhillon et al., 2004].

4.1.2 DNA damage considering bilirubin tertiles

Subjects were divided into tertiles with unconjugated bilirubin levels ranging from lowest to 11.12 µmol/L, from 11.29 to 20.69 µmol/L and from 20.86 µmol/L to the highest concentration. Classification into tertiles aimed to get more results concerning bilirubin concentration and DNA damage.

	Tertile1: 11.12 µmol/L	Tertile2: 11.29-20.69 µmol/L	Tertile3: 20.86 µmol/L	p-Value
Number	33	34	33	
MNC	0.33 ± 0.40	0.24 ± 0.36	0.24 ± 0.33	0.612
MN	0.41 ± 0.50	0.28 ± 0.39	0.27 ± 0.38	0.503
BE	0.70 ± 0.65	0.62 ± 0.62	0.45 ± 0.47	0.277
BNC	11.68 ± 3.99	11.44 ± 4.92	12.34 ± 4.93	0.714
CC/KR	9.81 ± 4.75	9.22 ± 5.48	9.93 ± 6.01	0.848
KL	36.64 ± 40.18	51.14 ± 67.08	36.19 ± 31.32	0.367
P	1.29 ± 0.88	1.26 ± 1.05	1.13 ± 0.83	0.770

Data are means ± SD.

Table 5: DNA damage represented in bilirubin tertiles

No significant differences were obtained between the tertiles, even though one might see higher levels of DNA damage in subjects with lowest UCB concentrations (Figure 15).

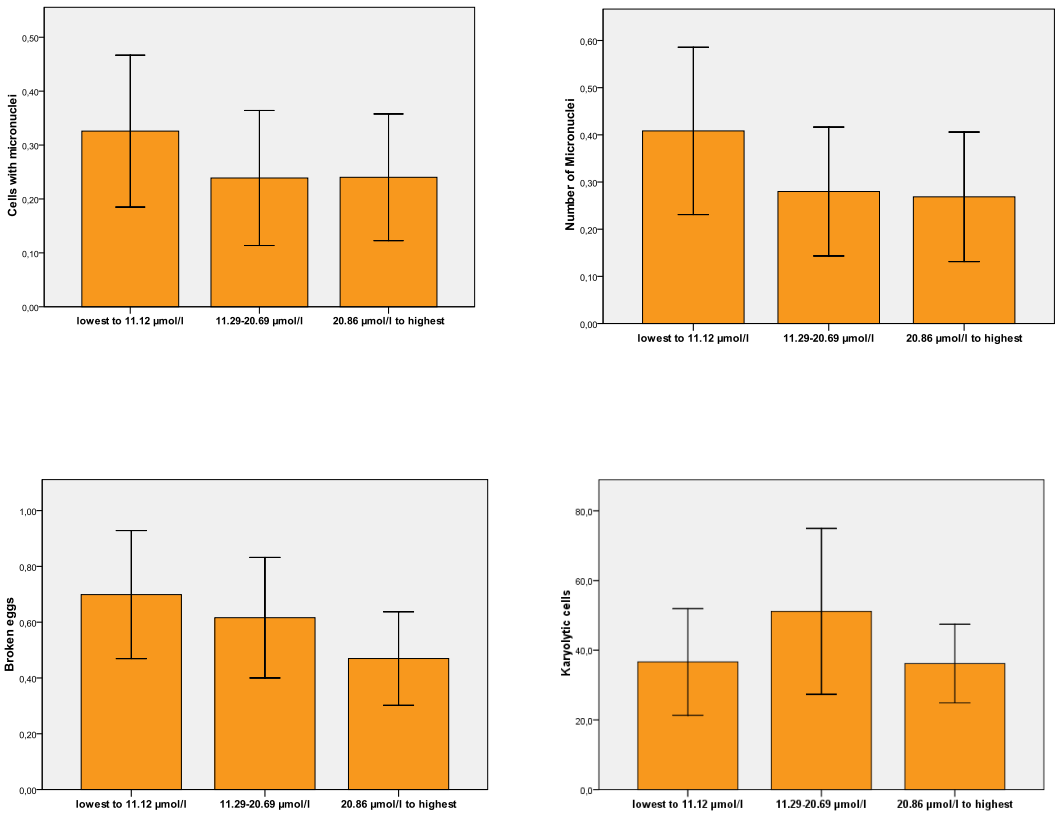


Figure 15: DNA damage represented in bilirubin tertiles

4.1.3 Factors influencing DNA damage

Gender

Data from the 70 male and 30 female subjects in the study were analyzed in order to find differences between both sexes (Table 6).

	Men	Women	p-Value
Number	70	30	
MNC	0.30 ± 0.37	0.20 ± 0.33	0.182
MN	0.34 ± 0.42	0.27 ± 0.45	0.315
BE	0.63 ± 0.60	0.52 ± 0.56	0.346
BNC	12.07 ± 4.49	11.22 ± 4.91	0.401
CC/KR	9.24 ± 5.35	10.60 ± 5.45	0.249
KL	35.80 ± 42.26	52.29 ± 60.19	0.088
P	1.23± 0.93	1.23 ± 0.93	0.977

Data are means ± SD.

Table 6: Influence of gender on DNA damage

No significant differences were discovered for parameters signalling DNA damage. However, when looking at mean values female subjects show fewer anomalies for almost all parameters. They had 33 % less cells with micronuclei, 21 % less micronuclei, 17 % less broken eggs and 7 % less BNC. A trend (p= 0.088) for less karyolysis in men was determined (see also Figure 16).

A study conducted by Fenech et al., 2005 showed that the amount of micronuclei in lymphocytes is generally higher in women than in men- and with age levels of MN are increasing progressively in both sexes. This goes along with other findings of our study that were described in thesis by Katharina Marisch and Maria-Theresia Pappenheim. Already proven for lymphocytes, for buccal epithelial cells clear information on gender differences cannot be given so far [Fenech, 2005, Marisch, 2011, Pappenheim, 2011].

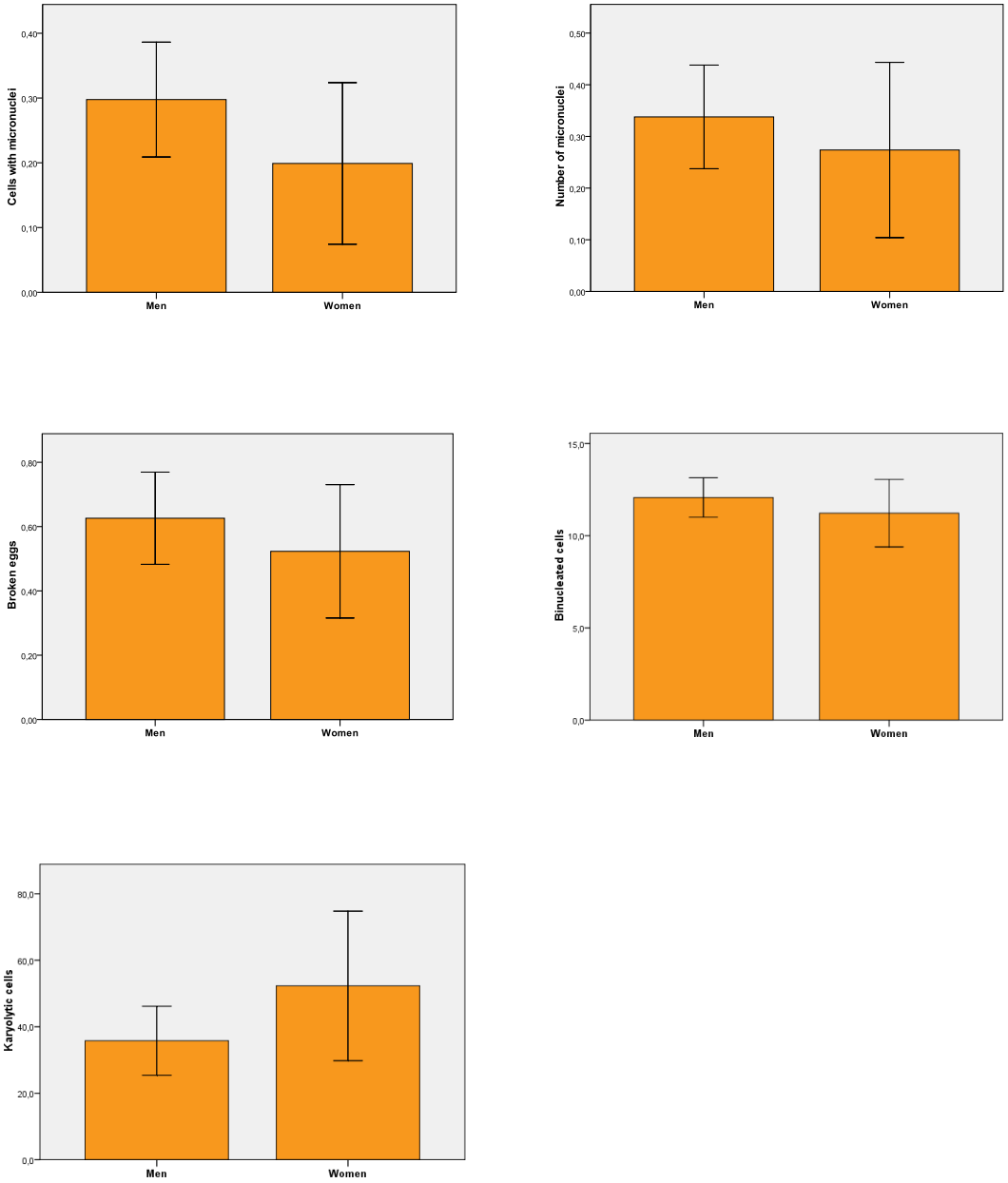


Figure 16: Gender differences concerning DNA damage

Age

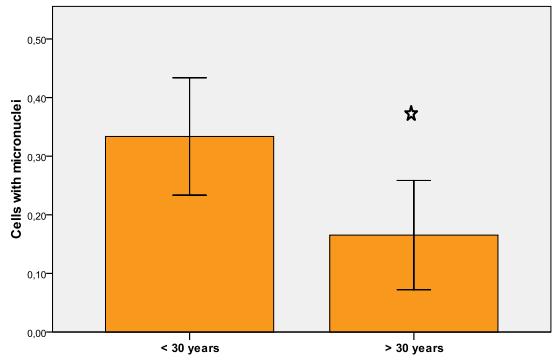
For evaluating the impact of age on the extent of oxidative DNA damage the individuals were divided into two different groups. Young subjects <30 years and old subjects ≥30 years. The results for mean DNA damage of these subgroups are shown in Table 7.

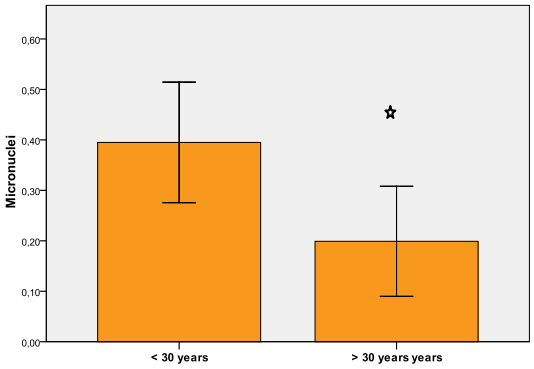
	<30 years	≥30 years	p-Value
Number	61	39	
MNC	0.33 ± 0.39	0.17 ± 0.29	0.024
MN	0.39 ± 0.47	0.20 ± 0.34	0.032
BE	0.73 ± 0.59	0.38 ± 0.51	0.001
BNC	12.63 ± 4.49	10.54 ± 4.57	0.027
CC/KR	10.57 ± 4.92	8.21 ± 5.83	0.040
KL	36.66 ± 44.05	46.29 ± 54.92	0.485
P	1.33 ± 0.87	1.07 ± 0.98	0.066

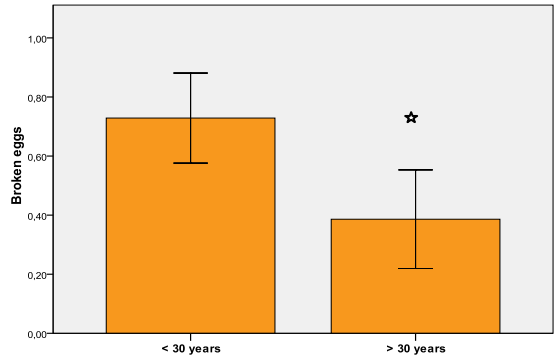
Data are means ± SD.

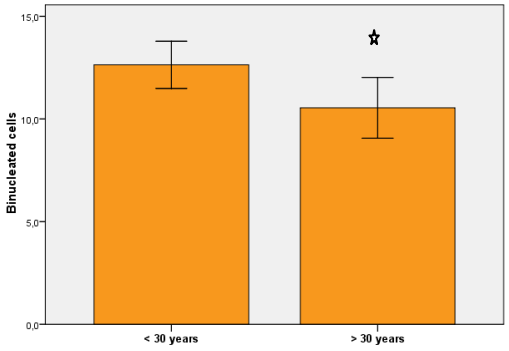
Table 7: Influence of age on DNA damage

Surprisingly, older individuals showed less numbers of cells with micronuclei, micronuclei, broken eggs, binucleated cells, condensed chromatin and karyorrhexis. A trend could be noticed for pyknotic cells, which were less in older subjects. Results are furthermore summarized in Figure 17.









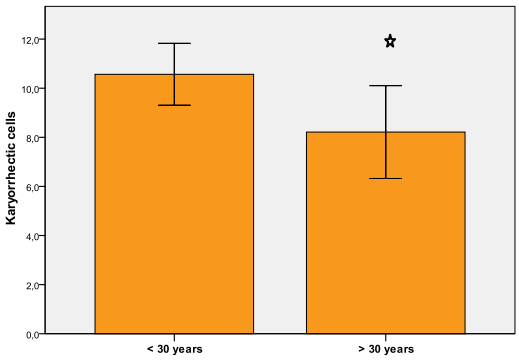


Figure 17: Influence of age on DNA damage

Age subgroups for GS and control

For more precise information the whole study population was later classified into younger (<30 years) and older (≥30 years) Gilbert´s Syndrome and control groups. Results are shown in the following figures.

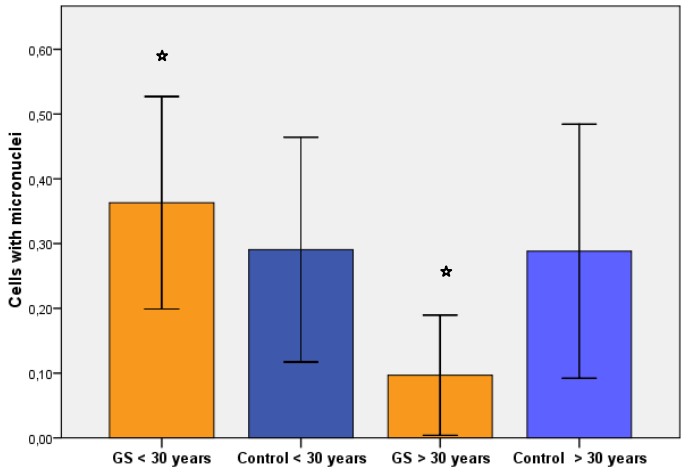


Figure 18: MNC: GS >30 years <GS <30 years (p <0.05)

Figure 18 shows that GS group (≥30 years) had significantly lower numbers of cells with micronuclei compared to GS group (<30 years; p<0.05).

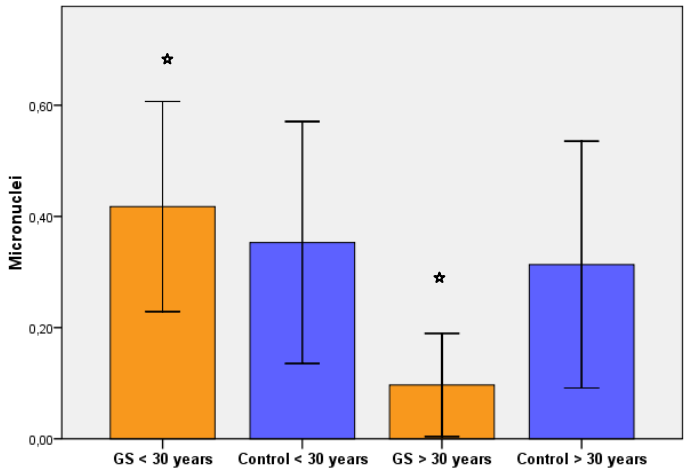


Figure 19: MN: GS >30 years <GS <30 years (p <0.05**)**

Figure 19 shows that GS group (≥30 years) had significantly lower numbers of micronuclei compared to GS group (<30 years, p<0.05).

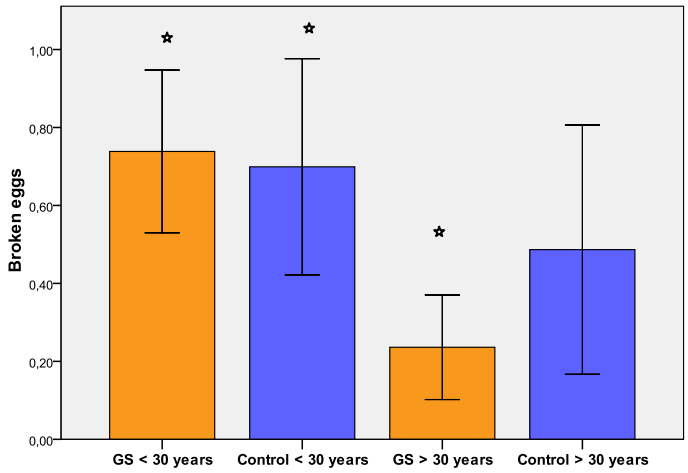


Figure 20: BE: GS >30 years <GS <30 years (p <0.05)

Figure 20 shows highly significant (p= 0.001) differences between older GS subjects compared to younger GS subjects and as well to younger controls (p= 0.001).

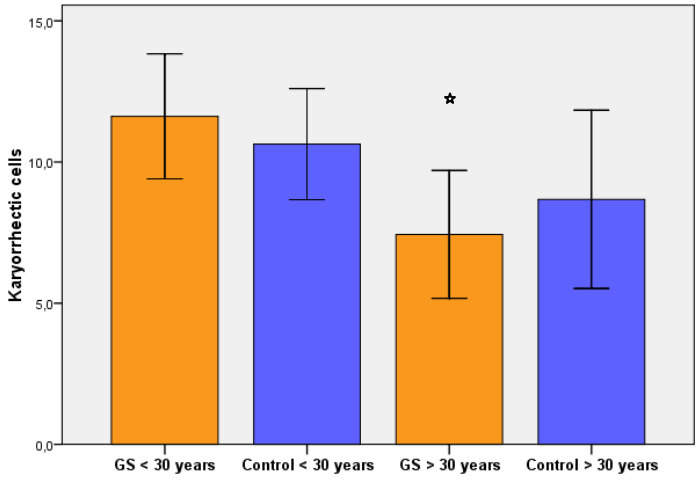


Figure 21: KR: GS <30 years >GS >30 years (p <0.05)

The number of karyorrhectic cells was significantly higher (p= 0.042) in older GS subjects than in younger GS subjects (Figure 21).

Allocation into two age groups showed significant differences between older and younger subjects and becomes clearer when examining these findings in GS. On the contrary Rickes, et al. were not able to determine any age effects in persons within 15 and 66 years [Rickes et al., 2010]. The values of MNC and

MN might have been influenced by UCB since younger and older controls did not show significant differences.

Due to a broad range of age (20-79 years) participants were divided into two age subgroups so that the effects of chronic exposure to elevated bilirubin on age related chromosomal damage could be determined. Subjects with GS ≥30 years had significantly lower numbers of MNC, MN and BE than the younger GS group. These are the first results maintained for chromosomal damage in buccal cells in GS that may indicate a protective effect against oxidative-stress related actions especially in individuals with chronically (>10 years) and moderately elevated UCB levels.

GS usually is not developed until adolescence due to an androgen steroid inhibition of the enzymatic bilirubin glucuronidation. This circumstance combined with the well documented antioxidative effects of UCB could be an explanation for protection from chromosomal damage in older individuals with GS [Muraca and Fevery, 1984].

Karyorrhectic cells by contrast were higher in GS subjects <30 years. KR is indicative for late stages of apoptosis. A possible hypothesis for an increased appearance of this cell type might be a higher cell division rate in younger people that makes elimination of useless or damaged cells necessary.

Furthermore, bilirubin induces apoptosis in colon cancer cells which is linked to a possible chemoprotective role in cancer development *in vivo.* Buccal and colon cells belong to epithelial lineages and therefore, these new findings in buccal cells might show an important pro-apoptotic role of bilirubin in chemoprevention [Keshavan et al., 2004].

4.1.4 Correlations

No significant correlations were obtained in the whole study population for UCB concentrations and DNA damage.

Correlations between older GS subjects and parameters of DNA damage with Vitamin B12 and folic acid did not show significant results. Folic acid is reported to have strong antioxidant potential and therefore might play an important role in the development of certain cancers and cardiovascular diseases [Stanger and Wonisch, 2012].

No investigation on the link between BMcyt-biomarkers and B vitamins has been performed yet. However, the results from a study by Stopper et al. showed that a combination of folic acid and Vitamin B12 was effective in lowering homocystein levels and numbers of MN in lymphocytes [Stopper et al., 2008].

5 CONCLUSION

The aim of the present study was to determine antigenotoxic effects of bilirubin. Moreover, an evaluation in individuals with chronically mild hyperbilirubinemia (Gilbert´s syndrome) was performed to detect whether this condition leads to a better protection against oxidative stress and oxidative stress mediated DNA damage.

In order to assess the antigenotoxic potential of bilirubin, biomarkers for chromosomal damage were measured in subjects with Gilbert´s syndrome and controls. The Buccal Micronucleus Cytome Assay (BMcyt) is a minimally invasive method for studying DNA damage, chromosomal instability and cell death. Numbers of Micronuclei (MN), cells containing MN (MNC) and broken eggs (BE) as well as other parameters like binucleated cells (BN), karyorrhexis (KR), condensed chromatin (CC), karyolysis (KL) and pyknosis were evaluated.

Bilirubin obtains strong antioxidative potential and therefore it was hypothesized that results show less DNA damage in subjects with GS compared to controls. This assumption could not been proved by performing this study. No significant differences for all parameters were found between GS and control group. Furthermore, no significant differences for all parameters were seen when dividing the study population into bilirubin tertiles.

Indeed, when dividing GS and controls into 2 subgroups of age (under and over 30 years), remarkable differences were discovered. Older GS subjects had significantly lower numbers of MN, MNC and BE than younger GS subjects. This reflects a better protection from genotoxicity in older individuals since they have profited from the chronic state of mild hyperbilirubinemia for a longer period of time (>10 years).

When looking at the total study population age aspects showed similar outcomes. Older participants had lower numbers of almost every measured parameter.

On the other hand no significant differences have been noticed regarding gender aspects.

Furthermore, when investigating the impact of folic acid and Vitamin B12 on DNA damage, no significant outcomes were obtained.

To conclude, a variety of different factors that influence biomarkers of oxidative DNA damage were investigated using the BMcyt. Influence was given through age, gender and also dietary substances like folic acid and Vitamin B12. It was not possible to detect the power of antioxidative properties of bilirubin towards a chromosomal protective effect in subjects with GS compared to controls. Despite all, the presented results could bring up the first indication for future research on people that are accumulating oxidative stress as seen in older participants. However, further research is needed for getting a better understanding of bilirubins´ antioxidant potential and its impact on DNA damage.

6 SUMMARY

Bile pigments are endogenous compounds that are actively produced in humans via the heme catabolism. The importance of bilirubin in humans has recently been demonstrated in large epidemiological investigations by protecting from cardiovascular events and cancer. However, so far no mechanistic data are available on the Buccal Micronucleus Cytome Assay (BMcyt), which is a minimally invasive method for studying DNA damage, chromosomal instability and cell death in high bilirubinic subjects (Gilbert´s syndrome).

In this case-control study with cross-sectional design, buccal samples were taken from 100 subjects, Gilbert´s syndrome and control. Allocation to the GS group required serum unconjugated bilirubin levels ≥17.1 µmol/L (controls <17.1 µmol/L). Buccal cells were collected from both cheeks of the participants. The obtained cells were extracted, fixed on slides and stained with light green (BMcyt). In addition to Micronuclei (MN) and cells containing MN (MNC) the number of other anomalies such as binucleated cells (BNC) and broken eggs (BE) as well as karyorrhexis (KR), karyolysis (KL), condensed chromatin (CC) and pyknosis (P) was evaluated.

It was hypothesized that subjects with increased UCB levels are better protected better against DNA damage. Results showed that there was no significant difference for all parameters between the GS and control group. However, when considering the age impact it was observed that BE ($p<0.05$) as well as MN and MNC ($p <0.05$) were lowest in the older GS group (subjects older than 30 years of age).

For the first time buccal cell damage was investigated regarding the bilirubin status in human subjects. Results showed that with increasing age the protective effects of bilirubin lead to less MN, MNC and BE which exhibits a first mechanistic prove of cancer protection by long-term elevated bilirubin levels in humans.

Nevertheless, only little information on the impact of bilirubin concentrations on DNA damage is available. Further research needs to be done for a better understanding of bilirubins´ protective effects, especially in risk groups of oxidative stress.

7 ZUSAMMENFASSUNG

Bei Gallenfarbstoffen handelt es sich um endogen produzierte Verbindungen, die im Menschen aktiv während des Häm-Abbaus produziert werden. Die Relevanz von Bilirubin wurde kürzlich durch große epidemiologische Untersuchungen festgestellt, die schützende Effekte auf kardiovaskuläre Erkrankungen und Krebs hervorhoben. Bis dato gibt es noch keine aussagekräftigen Daten für den Mikrokerntest in Mundschleimhautzellen (BMcyt), welcher eine minimal invasive Methode zur Feststellung von DNA-Schäden, chromosomaler Instabilität und Zelltod bei Personen mit erhöhten Bilirubinspiegeln (Gilbert´s syndrome) darstellt.

In dieser Fall-Kontroll-Studie mit Querschnittscharakter wurden Mundschleimhautzellen von 100 Teilnehmern, die entweder der Gilbert oder der Kontrollgruppe entsprachen, entnommen. Eine Zuweisung in die GS-Gruppe erforderte Werte von unkonjugiertem Bilirubin im Serum von ≥17.1 µmol/L (Kontrollen <17.1 µmol/L).

Die Mundschleimhautzellen wurden von beiden Wangeninnenseiten der Teilnehmer entnommen. Die gewonnenen Zellen wurden extrahiert, auf Objektträgern fixiert und mit Light Green gefärbt (BMcyt). Zusätzlich zu Mikrokernen (MN) und Zellen, die MN enthalten (MNC) wurden die Werte von weiteren Anomalien wie Binucleated Cells (BNC), Broken Eggs (BE), Karyorrhexis (KR), Karyolyse (KL), Condensed Chromatin (CC) und Pxknose (P) festgehalten.

Es wurde angenommen, dass Individuen mit erhöhten UCB-Werten besser vor DNA- Schäden geschützt werden. Laut den Ergebnissen konnten keine signifikanten Unterschiede für alle Parameter zwischen GS und Kontrollgruppe festgestellt werden. Allerdings konnte man bei näherer Betrachtung des Alterseinflusses beobachten, dass sowohl BE ($p<0.05$), als auch MN und MNC ($p<0.05$) in geringster Häufigkeit in der ältesten Gruppe auftraten (Teilnehmer älter als 30 Jahre).

Zum ersten Mal wurden Zellschäden in der Mundschleimhaut herangezogen um Rückschlüsse in Bezug auf den Bilirubinstatus in menschlichen Probanden zu

ziehen. Die Ergebnisse zeigten, dass mit zunehmendem Alter die schützenden Effekte von Bilirubin zum Tragen kommen, indem eine verminderte Anzahl an MN, MNC und BE festzustellen war. Dies stellt einen ersten mechanistischen Beweis für krebsschützende Effekte durch langzeitlich erhöhte Bilirubinspiegel im Menschen dar.

Nichtsdestotrotz gibt es nur sehr wenig Information bezüglich des Einflusses von Bilirubin auf DNA- Schäden. Es ist notwendig, weitere Untersuchungen durchzuführen, um ein besseres Verständnis der schützenden Effekte von Bilirubin zu erhalten, im speziellen für Risikogruppen die oxidativem Stress ausgesetzt sind.

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9 CURRICULUM VITAE

Simone Maria Blassnigg

Personal data

Date of birth	September 14th, 1986
Place of birth	St. Johann in Tirol
Citizenship	Austrian

Education

09/2005 - current	**Nutritional Sciences,** specialized in food technology University of Vienna
09/1997 - 06/2005	**Bundesgymnasium St. Johann in Tirol**
09/1993 - 06/1997	**Volksschule Fieberbrunn Dorf**

Experiences

10/2011 - 04/2012	**Schubert Apotheke** Department for Traditional Chinese Medicine
08/2010	**Saatzucht Edelhof**
11/2009 - 12/2009	**University of Vienna** Emerging Focus Nutrigenomics
07/2009	**Gebro Pharma GmbH** Laboratory for quality control
07/2008	**Gebro Pharma GmbH** Invoice processing
08/2009 - 10/2009	**Loomis Österreich GmbH**
07/2007	**Gebro Pharma GmbH** Invoice processing

International experiences

08/2009	**University of Vienna** Research in Siberia/ Russia Limnology, Vegetation and Soil Sciences

Languages

German	Mother Tongue
English	Fluent
French	Basic knowledge
Italian	Basic knowledge

EDV

WORD	Strong user skills
Excel	Basic user skills
SPSS	Basic user skills
EndNote	Basic user skills

Leisure

Sports (Running/Biking/Swimming/Hiking)
Reading novels, scientific magazines and travel literature
Travelling
Cooking for friends

Vienna, May 2012

Simone Maria Blassnigg