MASTERARBEIT | MASTER'S THESIS

Titel | Title

Effects of benign hyperbilirubinemia on Micronucleus frequency in buccal cells

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 838
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Ernährungswissenschaften
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Acknowledgement

Generating the results for this thesis was a team effort and could not have been done without the help of several people. First, I want to thank Birgit Stichaller, BSc for working with me on the method in the beginning of the process. Together we perfected the method and trained until steady results could be obtained. Secondly, I want to thank Rebekka Riepl, BSc for her scoring efforts toward the end of the process. Together we practiced through several weeks in order to obtain steady results for this thesis.

I also want to thank the working group of Univ.-Prof. Dr. Wagner for giving me the opportunity to work on this project and especially my personal supervisor Agnes Draxler, PhD. She consistently supported me throughout the whole project and never failed to answer any questions that came up during the process. I also want to thank her for her thoughtful comments and help on writing this thesis.

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Abbreviations

CRP	C-reactive-protein
DAPI staining	4’,6-diamidino-2-phenylindole staining
DMSO	Dimethylsulfoxid
BC	basal cell
BE	broken egg
BiliMetHealth	Bilirubin Metabolic Health
BLAVR	Biliverdin reductase
BMCyt	Buccal Micronucleus Cytome Assay
BN	binucleated cell
CC	condensed chromatin
DC	differentiated cell
HO	heme oxygenase
IBIL	indirect bilirubin (unconjugated bilirubin)
KH	karyorrhectic cell
KL	karyolitic cell
MN	micronucleated cell
MRP2	Multi drug resistance protein 2
NADP(H)	Nicotinamide adenine dinucleotide phosphate (hydrogen)
NAFLD	non-alcoholic fatty liver disease
NBUD	nuclear bud
NCD	non-communicable disease
OC	overlapped cells
PC	pycnotic cell
ROS	reactive oxygen species
rpm	rounds per minute
T2DM	type 2 diabetes mellitus
UC	uncountable cells
UDP	uridine diphosphate
UGT1A1	Uridine glucuronosyl transferase 1A1
WN	cell without a nucleus

1. Introduction

1.1. Gilbert Syndrome

Gilbert syndrome (GS) is defined as a condition that leads to an elevated level of unconjugated bilirubin in the serum or plasma. It is also referred to as benign hyperbilirubinemia or unconjugated hyperbilirubinemia. The prevalence of GS is approximately 5% of the general population. [Sanyal et al. 2018] However, the physiological range of serum/plasma bilirubin is not clearly defined. Thus, the prevalence in reported data ranges from 0,5% to up to 25,6%, with an average prevalence for the European population being 8,6%. [Wagner et al. 2018] Setting a cut-off-point for serum or plasma bilirubin is difficult because the bilirubin concentration can fluctuate due to various factors. However, GS is defined by the cut-off-point of 17.1 µmol/l bilirubin. Those factors influencing bilirubin concentrations include sex, ethnicity, age, smoking status, circadian rhythm, seasonal period, nutritional influences and physical activity. There is a large difference between men and women regarding serum and plasma bilirubin level, and this is the reason for a lower diagnostic cut-off-point for GS in women. [Vitek L., Tiribelli C. 2023]

Bilirubin is a yellow-orange bile product and a catabolic product of the heme metabolism. The heme catabolism results in biliverdin formation, before biliverdin is reduced to bilirubin. (figure 1)

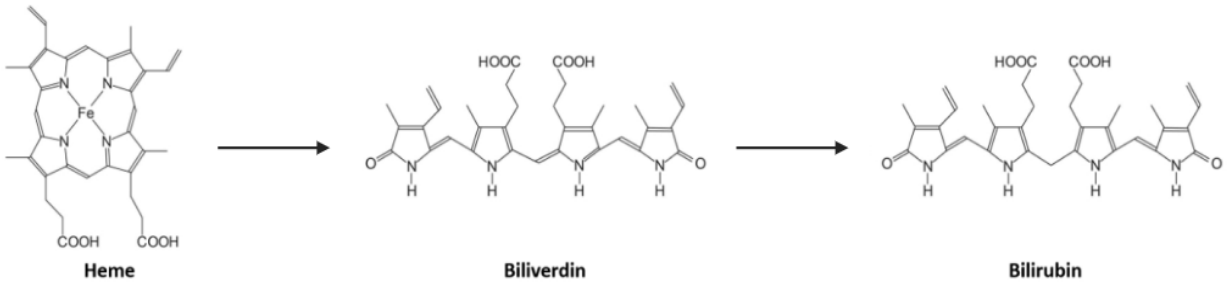

Figure 1: Important structures in heme catabolism [modified after Vitek et al. 2023]

Heme is liberated from red blood cells and catabolized by heme oxygenase to biliverdin releasing oxygen, iron and carbon monoxide. Afterwards, biliverdin reductase reduces

biliverdin to bilirubin by an NADPH to NADP mechanism. To be able to transport bilirubin into the bile canaliculus for excretion an additional metabolic step is required. This step includes the conjugation of bilirubin with glucuronic acid in the liver. Important enzymes required for those steps are the uridine glucuronosyltransferase 1A1 (UGT1A1) for conjugation and the Multi-Drug-Resistance-Protein 2 (MRP2) for excretion into the bile. In the next step of the pathway, the conjugated bilirubin is deconjugated again and can be excreted as stercobilin. A small share of the unconjugated bilirubin in the intestine is reduced to urobilinogen and reabsorbed into the enterohepatic circulation. (figure 2) [Ruiz et al. 2021] [Wagner et al. 2018]

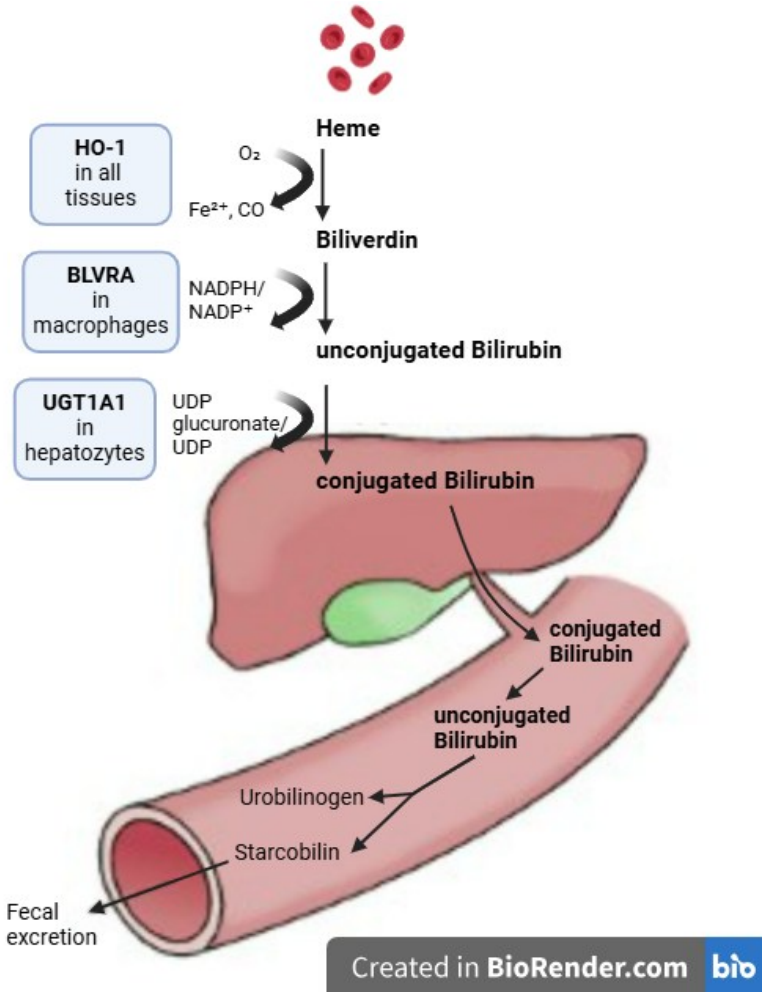

Figure 2: The bilirubin metabolism in the human body from heme through bilirubin to excretion. (modified after Ruiz et al.) Heme is enzymatically converted to biliverdin by liberating iron with loss of a carbon atom (CO). This opens the porphyrin ring, forms the open chain, which yields bilirubin after enzymatic reduction of biliverdin's central methine bond. In the liver, bilirubin is conjugated to enable excretion into the bile, requiring the enzyme UGT1A1. HO: Heme oxygenase; BLAVR: biliverdin reductase; UGT1A1: uridine glucuronosyltransferase 1A1 [Ruiz et al. 2021] [Wagner et al. 2018]. Modified after Ruiz et al. 2021 using Biorender.com

The most common reasons for benign hyperbilirubinemia are increased bilirubin production, impaired bilirubin uptake and impaired bilirubin conjugation. Frequently, a combination of those three pathophysiological conditions is the reason for hyperbilirubinemia to arise. Increased bilirubin production is a result of increased catabolic degradation of hemoglobin or other heme proteins. This can be accelerated by hemolysis, a hematoma, dyserythropoiesis or destruction of transfused erythrocytes. Impaired hepatic bilirubin uptake usually results from reduced hepatic blood flow or is induced by several drugs. In general, drug induced unconjugated hyperbilirubinemia typically resolves within 48 hours. Impaired bilirubin conjugation results from hereditary defects, including GS. GS is based on a decrease of UGT1A1 activity by 10-30% compared to normal activity. Functional impairments of the UGT1A1 enzyme inhibits the conjugation of bilirubin with glucuronic acid in the liver, which results in a mild or moderate hyperbilirubinemia. [Singh et al. 2023]

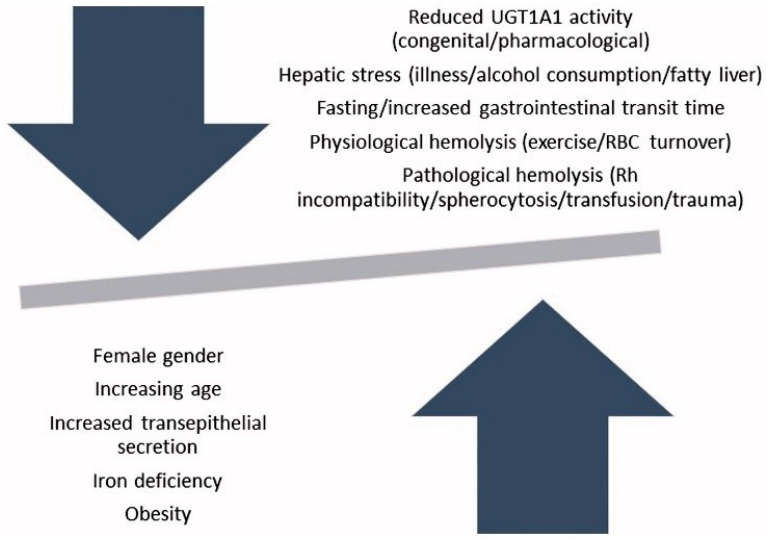

Figure 3: Factors affecting circulating unconjugated bilirubin (IBIL) concentrations [Wagner et al. 2018]

1.1.1. Antioxidant capability of unconjugated bilirubin

In the past, bilirubin was considered a waste product of the heme catabolism or even just a sign of liver disease or a potentially neurotoxic substance. Under certain conditions, very high levels of bilirubin can have neurotoxic effects and it can lead to elevated oxidative stress levels

in the cells. [Conti C. 2021] One example of bilirubin having neurotoxic effects is neonatal hyperbilirubinemia. In cases of neonatal hyperbilirubinemia, high levels of serum bilirubin can be potentially toxic to the central nervous system of infants. [Olusanya et al. 2018] It is suggested that bilirubin is involved in the balance between antioxidant and pro-oxidant agents. Despite the fact that bilirubin generally acts as an antioxidant, the relevance of the effects of bilirubin itself in processes causing neurotoxicity is still debated. [Dani et al. 2018]

Although severe hyperbilirubinemia is a considerable risk for morbidity and mortality and includes clinical symptoms like jaundice, behavioural impairments and cholestasis [Watchko, Tiribelli. 2013], “there is a second side of the coin”. More recent studies report various potential positive, health-promoting effects of mild hyperbilirubinemia. Those studies associate a mildly elevated bilirubin concentration in the blood with protection from cardiovascular diseases, type 2 diabetes mellitus (T2DM), some forms of cancers and even all-cause mortality. [Wagner et al. 2015] Some studies also link mild hyperbilirubinemia with lower obesity, a decreased risk of metabolic syndrome and a decreased risk of developing non-alcoholic fatty liver disease (NAFLD). [Vitek, Tiribelli. 2021] [Weaver et al. 2018]

In 2024 a secondary analysis of the data from the MARK-AGE cohort study was done by Schoissengeier et al. The data of 2489 participants was analysed and led to the conclusion that individuals with elevated unconjugated bilirubin concentrations are strongly associated with beneficial outcomes in oxidative stress parameters and metabolic health parameters. Those parameters include lower BMI, triglycerides, cholesterol, c-reactive-protein (CRP), insulin and higher HDL-cholesterol, glutathione and creatinine in individuals with higher unconjugated bilirubin concentration. The authors of this analysis conclude that elevated unconjugated bilirubin is a protective factor against cell damage. Those protective factors in turn affect the aging process and therefore life expectancy. [Schoissengeier et al. 2024]

The underlying process of these positive effects of bilirubin was long considered to be solely due to the antioxidant activities of bilirubin. However, some findings indicate that several additional and more biological potent activities could account for these beneficial effects that have been described for slightly elevated bilirubin concentrations. These biological potent activities of bilirubin include the modulation of cell signalling, protein phosphorylation, the

activation of cytoplasmic receptors, the activation of nuclear receptors and binding to molecules within the vascular bed and cell interior. Bilirubin thus seems to act as an endocrine molecule, with effects similar to those recently proposed for bile acids. Some authors therefore suggest using the term “bilirubinomics” to describe the whole field of study including the modulation of various cellular pathways. [Vitek, Tiribelli. 2021] [Gazzin et al. 2016]

It is also important to note, that those positive effects of bilirubin are contributed to unconjugated, indirect bilirubin (IBIL) and depend on the circulating concentrations of IBIL. For this reason, GS is often discussed and studied for its health benefits, since GS elevates the concentration of IBIL in the blood but only on a mild and health beneficial level. [Wagner et al. 2018]

1.2. Cell damage and Micronuclei in buccal cells

Buccal cells in the oral mucosa have a specific regenerative capacity in the human body. The regenerative capacity of tissues is fundamental for healthy aging. It is dependent on the number and division rate of the proliferating cells (basal cells), their genomic stability and their propensity for cell death. [Squier, Kremer. 2001] All of these events can be studied in buccal cells, which is an easily accessible tissue for sampling and minimally invasive, thus, leading to their increased use in human intervention studies. [Thomas et al. 2009]

There are two major outcomes, that can be looked at to determine the regenerative capacity of buccal cells. On one hand, by counting Micronuclei (MN), the level of chromosomal instability caused by underlying DNA damage can be assessed. On the other hand, the proportion of basal cells and cells undergoing cell death is an indication of the regenerative capacity of the buccal mucosa. [Thomas et al. 2009]

1.2.1. Parameters for chromosomal damage in buccal cells

The scoring criteria for cell types and nuclear anomalies in buccal cells is based on different categories that distinguish between “normal” cells and “abnormal” cells. These criteria are based on the original description of nuclear anomalies by Tolbert et al. [Tolbert et al. 1992] Cells are considered “abnormal” based on features that indicate DNA damage, cytokinetic failure or cell death. A summary of those features can be seen in figure 4. [Thomas et al. 2009]

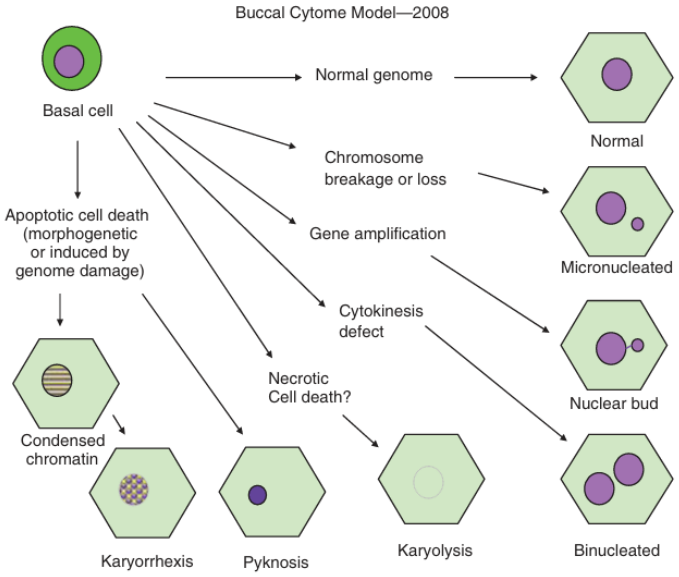

Figure 4: Buccal cytome (BMCyt) model – 2008 by Thomas et al. Diagrammatic representation of cell types scored in the BMCyt assay based on cytological and nuclear abnormalities. [Thomas et al. 2009]

Normal *differentiated cells* (DC) make up the largest number of cells counted in the buccal mucosa. They are characterized by a uniformly stained nucleus which is oval or round in shape. The difference between DC cells and *basal cells* (BC) is a larger nucleus to cytoplasm ratio in BC cells than DC cells and a darker stained cytoplasm in BC cells than in DC cells. [Bolognesi et al. 2013]

There are different forms of apoptotic cell death, including the cell types *condensed chromatin* (CC), *Karyorrhexis* (KH) and *Pyknosis* (PC). Apoptotic cell death can be morphogenetic or induced by genome damage. CC cells show a striated pattern in which the aggregated chromatin is intensely stained. In severe cases, the nucleus can appear fragmented. KH cells are characterized by even more extensive nuclear chromatin aggregation relative to CC cells.

The nucleus of KH cells has a densely speckled pattern. Pycnotic cells have nuclei that are small and shrunken with a high density of nuclear material that is uniformly but intensely stained. [Bolognesi et al. 2013]

Karyolitic cells (KL) are in a very late stage in the cell death process most likely induced by necrotic cell death. The nucleus of KL cells is completely depleted of DNA and not stained at all. [Bolognesi et al. 2013]

By counting those variations of cell death (CC, KH, PC and KL cells) and the frequency of BC cells in relation to "normal" DC cells a conclusion can be drawn to the regenerative capacity of the buccal mucosa. [Thomas et al. 2009]

Some other cell types that may also have relevance are *binucleated cells* (BN), cells with *nuclear buds* (NBUD) and cells with *broken eggs* (BE). NBUDs are small buds attached to the nucleus that might occur due to a budding process. The mechanism leading to NBUDs is not fully understood but may be related to the elimination of amplified DNA or DNA repair. BE cells are often categorized in the same category as NBUDs because of their similar appearance. The difference is a small thread seemingly connecting the bud and the nucleus. Normally the bud and the nucleus have the same size in BE cells, which only rarely happens in NBUD cells. The origin of BE cells and the underlaying mechanisms are uncertain. BN cells have two nuclei with the same size, morphology, texture and staining intensity. Most likely the reason for the formation of BN cells is a failure of cytokinesis either due to defects in formation of microfilament rings or cell cycle arrest due to for example telomere dysfunction. Therefore, the mononucleated to binucleated cell ratio is a useful biomarker indicative of cytokinesis failure and susceptibility to aneuploidy. [Bolognesi et al. 2013] Those cell types can be put into one category of cells undergoing some form of DNA damage including chromosomal breakage, gene amplification or defect cytokinesis. [Holland et al. 2008]

Figure 4 shows all types of cells that can be counted in the buccal mucosa. Frequencies of BE, KH, KL, PC and CC cells can provide information about cell death and therefore about the regenerative capacity of a tissue. On the other hand, counting BN, BE, NBUD and MN cells and

the frequency of those cells relative to “normal” DC cells can provide information about the extent of DNA damage and thus also the health of the mucosal tissue. [Holland et al. 2008]

1.2.2. Parameters for chromosomal damage: Micronucleated buccal cells

Micronucleated buccal cells (MN) are indicators of DNA damage. The formation of MN is the result of chromosomal breakage or chromosomal loss which can occur during early nuclear division due to chromosome or genome mutation [Hintzsche et al. 2017]. MN are formed following direct DNA damage or following secondary interactions with DNA replication apparatus. [Beedanagari 2017] Figure 5 shows the detailed process of micronuclear formation in lymphocytes. It indicates that a MN can be formed when a chromosome lags in anaphase, resulting in missegregation and exclusion from the main nucleus upon cytokinesis. This can either happen because of microtubule/kinetochore malfunction or when sister chromosomes are entangled throughout mitosis. [Nazaryan-Petersen et al. 2020]

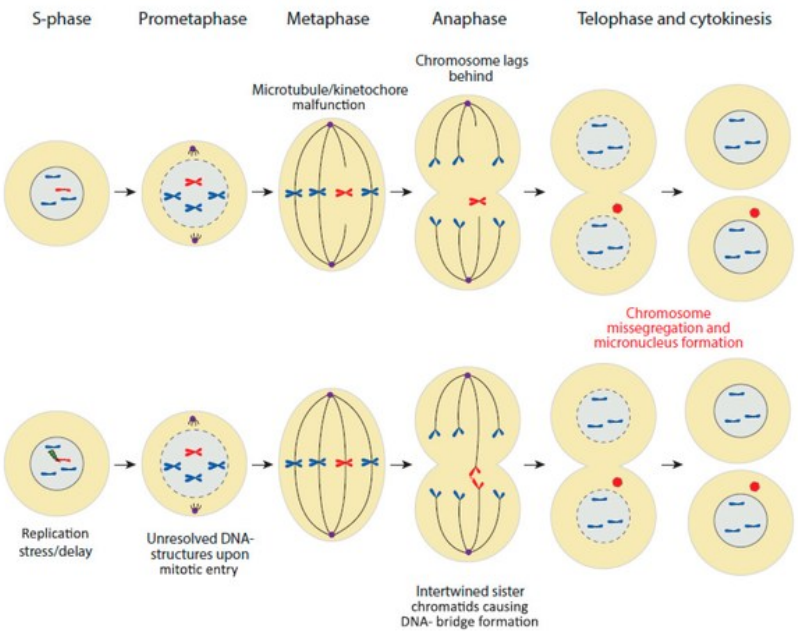

Figure 5: Mechanisms of micronucleus formation during mitotic cell division in lymphocytes. [Nazaryan-Petersen et al. 2020]

Cells with MN are characterized by the presence of both a main nucleus and one smaller nuclear structure. Most MN cells will contain only one micronucleus but in certain cases multiple micronuclei can appear in one cell, but this can rarely be detected. The MN have the

same staining intensity and texture as the main nucleus and must be located within the cytoplasm of the cell. [Thomas et al. 2009] [Bonassi et al. 2011]

Once a MN appears there are four major possibilities and two additional ones for the fate of the MN described in lymphocytes. The four major possibilities are degradation, reincorporation, extrusion and persistence. Based on current available knowledge it is concluded that the majority of MN persist at least until the next mitosis. Two additional possible fates of MN also need to be addressed and are premature chromosome condensation (chromothripsis) and elimination by apoptosis. (figure 6) The fate of MN differs depending on cell type, MN inducing agent and the species investigated. [Hintzsche et al. 2017]

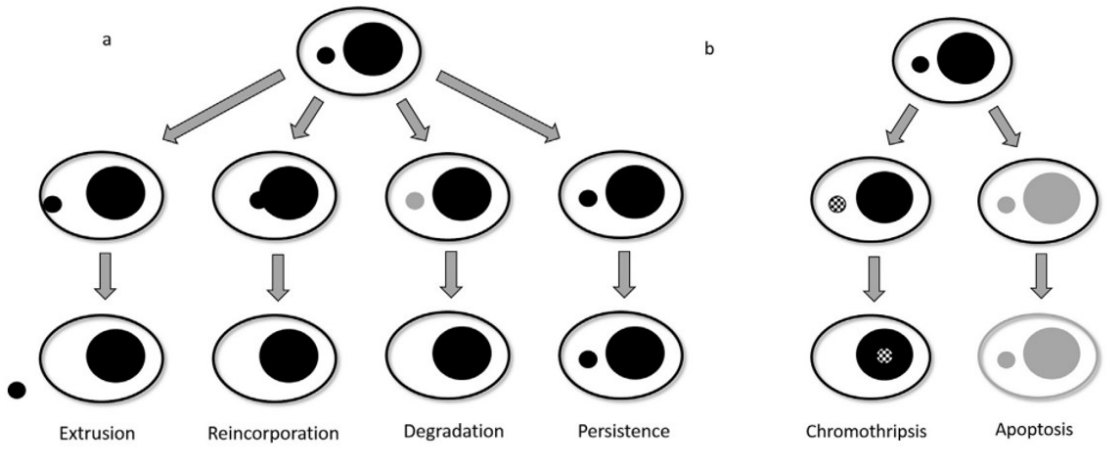

Figure 6: Schematic overview of the fates of a micronucleus. a) four major fates: extrusion, reincorporation, degradation and persistence; b) 2 additional fates: chromothripsis and apoptosis [Hintzsche et al. 2017]

Micronucleus frequency in lymphocytes is for example being used in biomonitoring studies as an indicator for exposure to genotoxins or as an indicator for genomic instability. Associations have for example been described between MN frequencies in lymphocytes and the cancer risk of a population. [Hintzsche et al. 2017] [Cardinale et al. 2012] In the past the MN frequency has mostly been used in peripheral blood mononuclear cells (PBMCs). However, several studies investigated MN frequencies in both lymphocytes and buccal cells and suggested a positive correlation in the two surrogate tissues. [Bonassi et al. 2011]

For instance, MN frequency in buccal cells has been shown to be a good indicator for genotoxic effects that can be the root cause of many different diseases. Many studies report using the buccal micronucleus cytome assay (BMCyt) to successfully show genotoxic effects of lifestyle factors. Those lifestyle factors include tobacco smoking and chewing, drinking alcohol, exposure to potentially mutagenic or carcinogenic chemicals and medical treatments, such as radiotherapy. [Holland et al. 2008] [Stich, Rosin. 1983] Genomic instability biomarkers such as MN frequency are also elevated with advancing age. Additionally, MN frequency in buccal cells has been theorized to even more accurately reflect age-related genomic instability than other cell types. [Thomas et al. 2008]

Some studies also investigated the effect of nutrition, specifically micronutrient deficiencies and micronutrient supplementation on MN frequency in buccal cells. Those studies show that using single micronutrient supplements like B12 or folic acid might improve chromosomal instability parameters in buccal cells measured with the BMCyt. Some other micronutrients showing success in reducing MN frequency in buccal cells include vitamin E, vitamin A and beta-carotin. Nutrient deficiencies such as B12 and folic acid deficiencies also seem to affect MN frequency in buccal cells negatively. The data from those same studies also suggests that firstly, multiple micronutrient combinations are more efficient in reducing MN frequency than individual micronutrients, under conditions of normal micronutrient status. Secondly, micronutrients were most efficient in reducing MN frequency when the initial baseline level of those micronutrients was insufficient. [Thomas, Wu et al. 2011]

2. Relevance of this thesis

2.1. Aim of this thesis

This thesis investigates whether an elevated level of unconjugated bilirubin (IBIL) in the blood influences the MN frequency in buccal cells. Specifically, individuals with GS were investigated because GS leads to an elevated level of IBIL in the blood. [Sanyal et al. 2018] Research suggests that slightly elevated levels of IBIL have multiple positive effects including less chromosomal damage caused by DNA damage in lymphocytes. [Wallner et al. 2012] However, chromosomal damage parameters can also be investigated in buccal cells and investigating them in form of MN frequency in buccal cells gained popularity in recent years due to the quick and simple sampling of buccal cells and because studies suggest that there is a positive correlation between MN frequency in buccal cells and lymphocytes. [Thomas et al. 2009] [Sommer et al. 2020]

To evaluate if IBIL levels have an influence on MN frequency in buccal cells a total of 46 subjects with GS and an equally big control group were investigated using data and samples from the Bilirubin Metabolic Health Study (BiliMetHealth). Similar studies on this topic would suggest the hypothesis that the MN frequency should be lower in subjects with GS compared to subjects without GS due to antioxidant and metabolic effects of IBIL. [Bulmer et al. 2008] [Conti C. 2021] This is why the aim of this study is to compare MN frequency in buccal cells of subjects with GS with their control group to investigate whether IBIL makes a significant difference on chromosomal damage parameters in buccal cells.

2.2. Current status of research

Over time, various studies reported positive effects when investigating subjects with GS. Those positive, health promoting effects can be attributed to the elevated bilirubin concentration in subjects with GS. Elevated bilirubin concentrations for example exert a protective role against various non-communicable diseases (NCDs), including various forms of cancers, respiratory diseases, NAFLD, T2DM and cardiovascular diseases. [Horsfall et al. 2011] [Vitek, Tiribelli. 2021] Some studies also link mild hyperbilirubinemia with lower adiposity and a decreased

risk of metabolic syndrome. [Weaver et al. 2018] This coincides with other studies that suggest that a mild hyperbilirubinemia is even associated with protection from all-cause mortality. [Wagner et al. 2015]

In 2024 a secondary analysis of the data from a cohort study called “MARK-AGE” was published by Schoissengeier et al. showing even more detailed analysis of the positive effects that an elevated bilirubin concentration can have on metabolic health parameters. In total, the data of 2489 participants was analysed and led to the conclusion that individuals with elevated unconjugated bilirubin concentrations are strongly associated with beneficial outcomes in oxidative stress parameters and metabolic health parameters. They found that subjects with elevated bilirubin concentrations, in comparison to subjects with no elevated bilirubin, are associated with lower BMI, triglyceride, cholesterol, CRP and insulin in the blood. Moreover, high bilirubin concentrations were associated with higher HDL-cholesterol, glutathione and creatinine in comparison to individuals with lower bilirubin concentrations. Those mentioned metabolic health parameters and oxidative stress parameters can influence cellular changes associated with aging. Authors of this analysis therefore conclude, that elevated unconjugated bilirubin is a protective factor against cell and DNA damage. Thus, bilirubin can positively influence cellular changes associated with aging and can have an effect on aging processes and therefore life expectancy. [Schoissengeier et al. 2024]

The underlying physiological reasons for these protective mechanisms include antioxidant properties of bilirubin as well as the whole “bilirubinomics” system. The field of the so called “bilirubinomics” investigating all the possible effects of bilirubin on the human body and its cellular pathways is relatively new. It entails the hypothesis that several additional and more biological potent activities than the antioxidant activities of bilirubin account for the beneficial effects that have been described for slightly elevated bilirubin concentrations. These biological potent activities of bilirubin include the modulation of cell signalling, protein phosphorylation, the activation of cytoplasmic receptors, the activation of nuclear receptors and binding to molecules within the vascular bed and cell interior. Bilirubin thus seems to act as an endocrine molecule, with effects similar to those recently proposed for bile acids. Some authors therefore suggest using the term “bilirubinomics” to describe the whole field of study

including the modulation of various cellular pathways. [Vitek, Tiribelli. 2021] [Gazzin et al. 2016]

Not many studies have been done on chromosomal instability parameters in buccal cells in association with bilirubin concentrations so far. In 2012 Wallner et al. investigated the effects of bilirubin on chromosomal damages in buccal cells. The data of 76 subjects was analysed to determine the effect of high bilirubin concentrations on chromosomal instability parameters in buccal cells. To analyse and score the buccal cells the BMCyt method by Thomas et al. 2009 was used. No significant differences between GS group and control group for all endpoints of buccal cell chromosomal damages were found. However, the authors conclude that DNA instability is decreased in older subjects with GS compared to older subjects without GS. These findings might indicate that GS subjects are protected against the consequence of variation in the genetic material. [Wallner et al. 2012] Although the effect of age on chromosomal damage parameters in lymphocytes is well documented in the literature, studies investigating buccal cells do not always find a clear effect of age on buccal cell chromosomal damage parameters. [Rickes et al. 2010] [Ceppi et al. 2010]

3. Materials and Method

3.1. Study design

The samples for this study were obtained as part of the Bilirubin Metabolic Health study (BiliMetHealth). BiliMetHealth is a case control study carried out at the University of Vienna within an FWF project. A total of 92 subjects were enrolled in the study. Cases are individuals with GS. The participants in the control group were age (± 2 years) and gender matched to the cases.

The BiliMetHealth study focuses on the whole metabolomic system in comparing metabolomics from the GS group to the control group to get an overall picture of the health benefits of a slightly elevated bilirubin status.

Inclusion criteria for both groups were an age between 18 and 65 and physical activity about 2 times per week. Exclusion criteria are as follows: frequent and regular smoking, chronic illnesses (T2DM, metabolic syndrome, heart diseases), pregnancy and breastfeeding, hepatic diseases (including Hepatitis A and B), renal diseases, any form of tumours, organ transplants and unstable body weight. As for the bilirubin levels the inclusion and exclusion criteria must be different for the case compared to the control group. Inclusion criteria for cases with GS is a level of unconjugated bilirubin in the blood over 1 mg/dl and total bilirubin over 1,2 mg/dl, whereas for the control group the level of unconjugated bilirubin in the blood needs to be beneath 1 mg/dl and the total bilirubin level beneath 1,2 mg/dl. More detailed information can be seen in table 1.

	inclusion criteria	exclusion criteria
case group - Gilbert´s syndrome	total bilirubin: > 1.2 mg/dl IBIL: > 1.0 mg/dl	total bilirubin: ≤ 1.2 mg/dl IBIL: ≤ 1.0 mg/dl
control group	total bilirubin: ≤ 1.2 mg/dl IBIL: ≤ 1.0 mg/dl	total bilirubin: > 1.2 mg/dl IBIL: > 1.0 mg/dl

Table 1: Inclusion and exclusion criteria for subjects of the BiliMetHealth study differentiated according to Gilbert´s syndrome case or control group. IBIL - indirect bilirubin, unconjugated

The study subjects are subsequently also undergoing an exercise challenge but this outcome is not considered in this master thesis. Buccal cells have been sampled from all study subjects at baseline and the comparison for the results is based on a comparison of cell damage in buccal cells between the case group (GS) and the control group (gender and age matched) without intervention.

3.2. Buccal micronucleus cytome assay (BMCyt)

One method to examine chromosomal instability caused by underlaying DNA damage, cell death and regenerative potential of human buccal cells is called the *Buccal micronucleus cytome assay (BMCyt)* using the Feulgen staining method to stain buccal cells. [Thomas et al. 2009] To investigate buccal cells, different variations of the BMCyt and different staining methods have been used in the past. Therefore, the results of different studies conducted and analysed between different laboratories should not be compared entirely. Besides the Feulgen staining method some other popular staining methods are the Giemsa staining, acridine orange and the 4',6-diamidino-2-phenylindole (DAPI) staining method. [Nersesyan et al. 2006] [Holland et al. 2008]

In an effort to standardise the method of the BMCyt Thomas et al. published a protocol in 2009 which includes the sampling and staining of the buccal cells and the scoring criteria of the BMCyt parameters. The mentioned protocol uses the Feulgen staining method with Schiff´s reagent in order to stain the nucleus and Light Green to counterstain the cytoplasm. The primary goal of this staining method is to ensure that the results from future studies could be compared better and to achieve results of high quality. [Thomas et al. 2009]

After extensive research the BMCyt protocol of Thomas et al. was chosen as the method for the analysis of buccal cells collected in the BiliMetHealth study. [Thomas et al. 2009] However, the method was optimized by using different laboratory equipment and procedures.

The BMCyt is comprised of four major steps. Those steps include 1) sampling, 2) slide preparation, 3) staining and 4) scoring. Step number one is the sampling of buccal cells, which is carried out by using a toothbrush to scrape buccal cells from the cheek. Following this, the

sampled cells are being washed multiple times with a buccal cell buffer using multiple rounds of centrifugation and removal of the supernatant. In step number two the cells need to be counted and diluted to 80.000 cells per ml before they can be put onto slides using a cytocentrifuge. Once the cells are on the slide they need to dry for exactly 10 minutes. Afterwards the slides are being washed by putting them for 1 minute into 50% ethanol followed by 1 minute in 20% ethanol and finally for 2 minutes in milli-Q-water. After the washing steps the slides are incubated with 5 M HCl for 30 minutes. HCl is necessary to break already open nuclear structures to be stained. [Thomas et al. 2009]

Step number three is the staining of the cells using the Feulgen method. When the incubation time in 5 M HCl is over the slides are rinsed under running water for 3 minutes to get rid of the HCl solution. Afterwards the cells are stained in Schiff´s reagent for 60 minutes in the dark. Next, the Schiff´s reagent needs to be washed off by running water for 5 minutes. Lastly, the cytoplasm is counterstained by using light green for 30 seconds. Immediately after those 30 seconds the green needs to be washed off thoroughly. [Thomas et al. 2009]

Before step number four, which is the scoring of different cell types using a light microscope at 1000x magnification, can begin, cover slips need to be placed onto the slides. The slides need to be completely dry before a drop of DePex is used to put the cover slips onto the slides. Afterwards they need to dry under a fume hood for about 24 hours or at least overnight. [Thomas et al. 2009]

Step number four is the scoring of different cell types. Figure 7 shows a flow chart of the exact scoring process used in the BMCyt by Thomas et al. 2009. [Thomas et al. 2009]

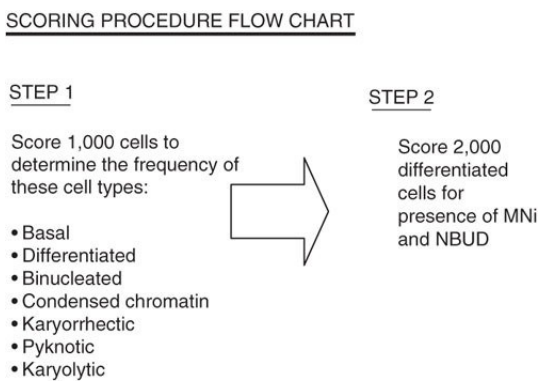

Figure 7: Flow chart showing the scoring criteria from the BMCyt [Thomas et al. 2009]

3.2.1. Changes and adjustments of the protocol to Thomas et al. 2009

In this study, there were a few adjustments to the original protocol from Thomas et al. 2009 made. A summary of all the adjustments can be found in chapter 3.3.

The sampling of buccal cells for the BiliMetHealth study was carried out in accordance with the protocol by Thomas et al. 2009. Afterwards, the cells were stored in methanol at -80°C until further analysis. [Szczepek et al. 2019] The cells being stored in methanol at -80°C is the biggest difference to the original protocol, where the slide preparation starts immediately after sampling. [Thomas et al. 2009]

Compared to the original protocol by Thomas et al. 2009, in the first step the frozen samples had to be thawed before the cells were filtered through a nylon-membrane-filter “milipore” (pore size 100,0 µm) using a syringe. In contrast to the original protocol the homogenization of the buccal cells was not carried out.

In the protocol of Thomas et al. 2009, a different cell counter was used. This is why the preparation of the cells for the cell counter varies from the one in the protocol. [Thomas et al. 2009] For the BiliMetHealth study 10µl of Trypan blue is mixed with 10µl of the sample and after carefully mixing the dilution with a pipette 10µl were used to perform the cell count. After diluting the sample to 80.000 cells per ml it was apparent that this dilution often did not sufficiently separate the cells from one another. The separation of the cells on the slides however is essential to achieve cells that do not overlap and therefore can be counted. Hence, the buccal cells in the BiliMetHealth study were diluted using the double volume of buccal cell buffer and dimethyl-sulfoxide (DMSO), leading to optimized results on the microscope slide in terms of cell number and countability.

For the process of transferring the cells onto the microscope slides a cytocentrifuge using 800rpm for 5 minutes with a medium acceleration was used.

3.2.2. Changes and adjustments of the staining process to Thomas et al. 2009

The first steps were not altered and applied as described in the original protocol. For the staining process the slides were incubated for 10 minutes in ethanol:glacial acetic acid (3:1) in order to fixate the cells onto the slides. Afterwards the slides were dried for 10 minutes before the washing steps began. The washing steps started with 1 minute in 50% ethanol before 1 minute in 20% ethanol and finally 2 minutes in milli-Q-water. After the washing steps were completed, the slides were incubated for 30 minutes in HCl to break the nuclear structures for the staining process. Afterwards, the slides were washed for 3 minutes under running tab water. [Thomas et al. 2009]

The next staining step was the incubation in Schiff´s reagent for a 50 minute staining duration. Afterwards, the slides were washed for 5 minutes under running water.

For counterstaining a fast green solvent was used, consisting of a 0,2% fast green stock solution. This stock solution was diluted 1:5 with milli-Q-water right before it was used. The optimal time for counterstaining in fast green was approximately between 8-10 seconds. Afterwards, the green colour was washed off immediately and very precisely. [Yang et al. 2017] [Torres-Bugarin et al. 2014]

Figure 8 shows the different staining methods that were compared and analysed during the preparation for the study. Picture f within figure 7 shows the final alteration, with the best colouration of the cytoplasm and the most visible structures in the nucleus. It is necessary to see the structure and patterns in the nucleus for precise scoring.

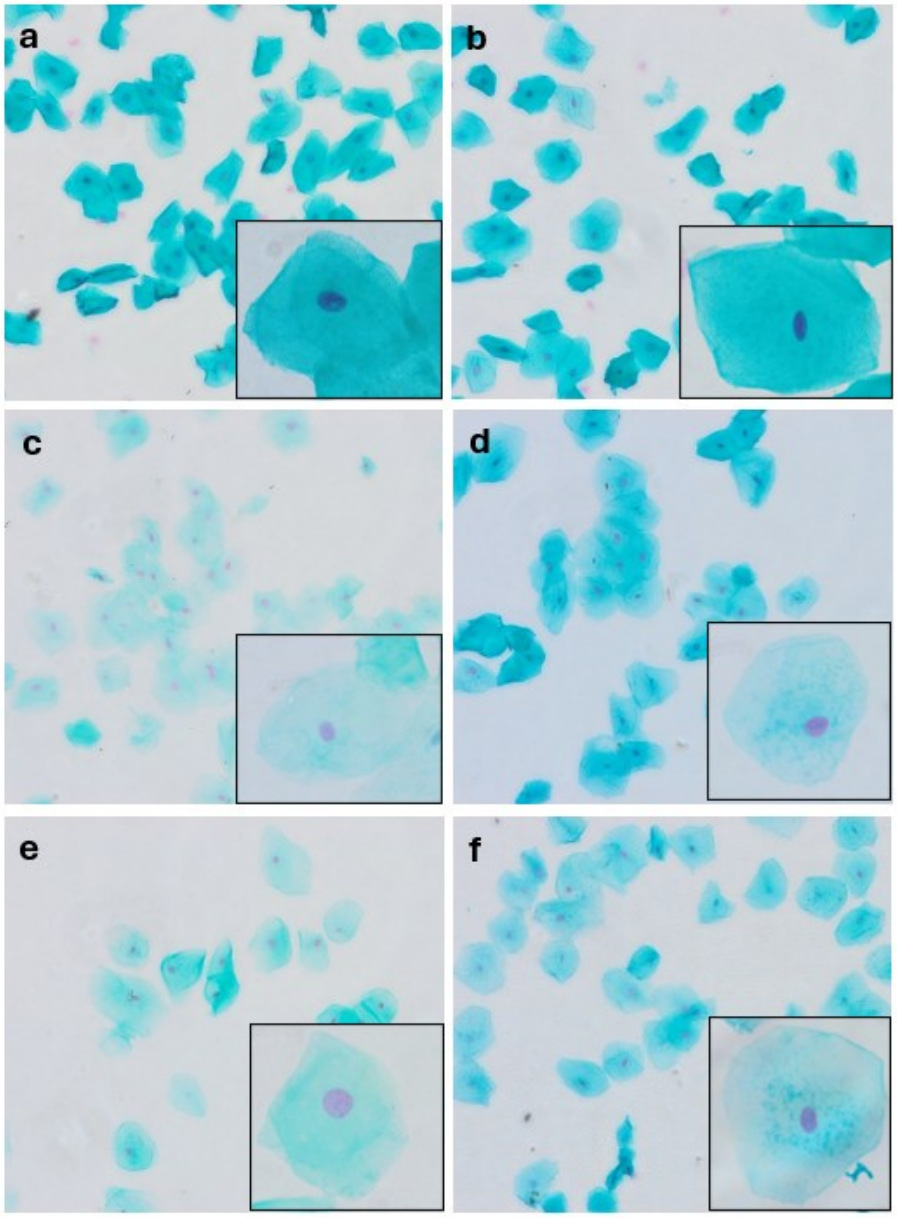

Figure 8: Images of buccal cells prepared and stained using the BMCyt but using different variations of the Feulgen staining method. Pictures taken under transmitted light in 400x und 1000x magnification. a) Schiff´s reagent 60min, fast green 30sec (0,2%, 5:1 diluted, without glacial acetic acid); b) Schiff's reagent 60min, fast green 10sec (0,2%, 5:1 diluted, without glacial acetic acid); c) Schiff´s reagent 60min, fast green 10sec (0,1%, not diluted) with glacial acetic acid; d) Schiff´s reagent 60min, light green 10sec (0,1%, not diluted, with glacial acetic acid); e) Schiff´s reagent 90min, light green 30sec; f) Schiff´s reagent 50min, fast green 8-10sec (0,2%, 5:1 diluted, with glacial acetic acid)

3.2.3. Changes and adjustments of the scoring criteria to Thomas et al. 2009

A new separate counting category was implemented for all cells that overlapped to estimate the number of cells that could not be taken into account. The occurrence of overlapped cells might be a result of the lack of homogenization after sampling due to a missing homogenizer. Thus, two subgroups for this Master thesis were created called *overlapped cells* (OC) and *uncountable cells (UC)*. *not countable cells (UC)* are cells that are unable to be counted because of the form of their cytoplasm, form of their nucleus or because of an object or air bubble in front of it. [Thomas et al. 2009] [Bolognesi et al. 2013]

On the other hand, another new subgroup was added for cells completely *without a nucleus (WN)*, since numerous cells were missing their nucleus completely. A reason for the cells with an absent nucleus should be investigated further.

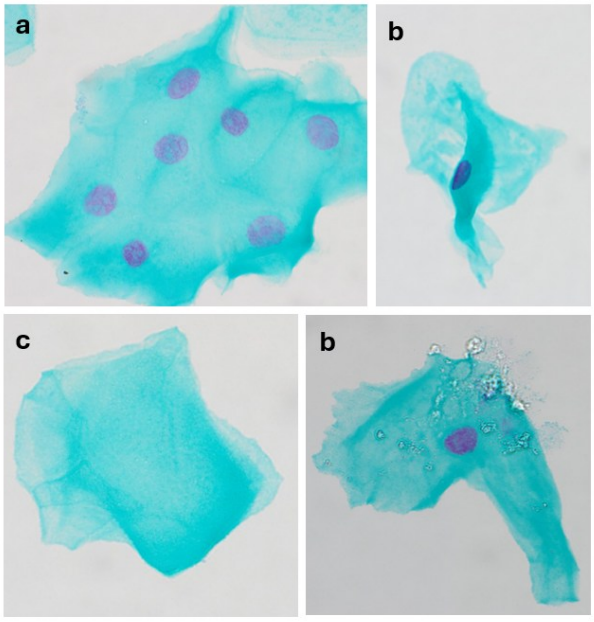

Figure 9: Different categories of uncountable buccal cells prepared and stained using the BMCyt. Images taken using a transmitted light microscope with a 1000x magnification. a) overlapped cells (OC) that cannot be counted due to no clear beginning or end of the cytoplasm; b) uncountable cells (UC) that cannot be counted due to the form of their cytoplasm, form of their nucleus or objects in front of it; c) cells without a nucleus (WN) cannot be counted because of the complete missing of the nucleus or any shade of it

3.3. Summary of the adjustments of the BiliMetHealth protocol

Table 2 shows all the detailed modifications that were undertaken to form a slightly different protocol for preparation and staining of buccal cells for the BiliMetHealth study.

nature protocol [Thomas et al. 2009]	adjustments
slide preparation and staining directly after sampling	storing the samples in methanol (80%) at -80°C after sampling, slide preparation and staining at a different time
homogenize the cell dilution	no homogenizing of the dilution because no homogenizer was available
sample dilution in 1ml buccal cell buffer for cell count	sample dilution in 0,5ml buccal cell buffer for cell count
sample dilution to 80.000 cells per ml before cytocentrifuge	sample dilution to 40.000 cells per ml before cytocentrifuge
cytocentrifuge at 600rpm for 5min	cytocentrifuge at 800rpm for 5min at medium acceleration
staining the slide in Schiff´s reagent for 60 min	staining the slide in Schiff´s reagent for 50 min
counterstaining with light green for 20-30 seconds	counterstaining with fast green (0,2% with glacial acetic acid), diluted 1:5 with millli-Q-water for 8-10 seconds
2 drops of DePex for the coverslip	1 small drop of DePex for the coverslip

Table 2: Summary of the detailed modifications from the original nature protocol (Buccal micronucleus cytome assay by [Thomas et al. 2009]) for the purposes of the BiliMetHealth study. ml = millilitre; rpm = rounds per minute

3.4. Adjustments of the scoring criteria

For the scoring of the buccal cells a light microscope was used at x1000 magnification. In a first step, the different cell types in 1000 cells were counted. Those cell types are differentiated cells (DC), basal cells (BC), condensed chromatin (CC), pycnotic (PC), karyolitic (KL), karyorrhectic (KH), binucleated cells (BN), broken eggs (BE), nuclear buds (NBUD) and micronuclei (MN). cells without nucleus (WN), cells overlapping with others (OC) and uncountable cells (UC) are also counted but not considered for the 1000 counted cells.

In step 2, 1000 more cells are counted to obtain the count of MN in 2000 cells. In this step, the MN in 1000 countable cells are taken into account. Countable cells exclude the categories WN, OC and UC. [Bolognesi etl.al. 2013] (figure 10)

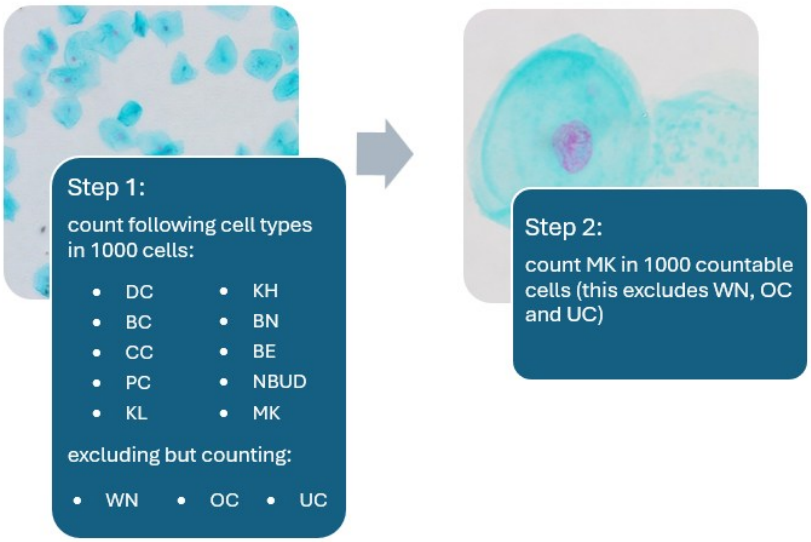

Figure 10: Flow chart showing the adjusted process of scoring buccal cells for this thesis. DC - differentiated cell; BC - basal cell; CC - condensed chromatin; PC – pycnotic; KL - karyolitic cell; KH - karyorrhectic cell; BN - binucleated cell; BE - broken egg; NBUD - nuclear bud; MN - micronucleated cell; WN - without nucleus; OC - overlapping cells; UC - uncountable cells

3.5. Statistical analysis

The scoring results were imported to the IBM SPSS software (version 28.0.0) and the data was prepared for analysis. The two groups being analysed and compared are the case group with GS and the control group. The normal distribution of the data was tested using the Shapiro-Wilk test. If normal distribution was given, an independent t-test was performed. The Mann-Whitney-U-test was used for not normally distributed data. For detecting correlation of two parameters Pearson, Chi^2 and Eta was used, depending on the scalation of the parameters.

The comparison of the MN frequency between the two groups is controlled for age and gender. This was carried out using partial correlation for an overview of the data and afterwards a grouped Mann-Whitney-U-test to test for significance. For stating the significance $p < 0.05$ was used.

4. Results

4.1. Analysis of chromosomal instability parameters

A total of 92 sets of data from the BiliMetHealth study were included in this analysis. Table 3 shows the general description of the data comparing GS case and control groups. (table 3)

		Gilbert syndrome		
		YES	NO	**total**
age	18-34 years	23	25	48
	34-63 years	23	21	44
total		46	46	92
sex	f	19	18	37
	m	27	28	55
total		46	46	92
BMI	underweight	1	2	3
	normal weight	39	29	68
	overweight	6	11	17
	obese	0	3	3
total		46	45	91

Table 3: General description of the data. Frequency table grouped after Gilbert syndrome case and control group. f - female, m – male; underweight: BMI < 18,5 kg/m²; normal: BMI 18,5-24,9 kg/m², overweight: 25-29,9 kg/m², obese: BMI >30 kg/m²

Comparing the MN frequency in 2000 countable buccal cells there is no significant difference between the GS group and the control group. ($p = 0.135$) When looking at figure 10, a slightly but not significantly higher mean MN count can be seen in the control group compared to the case group. The mean MN count in the case group is 0.636 (± 0.209) MN in 2000 buccal cells whereas it is 0.930 (± 0.272) in the control group. Although no significant difference could be found between the two groups, a tendency of slightly more MN cells in the control group can be seen. (figure 11)

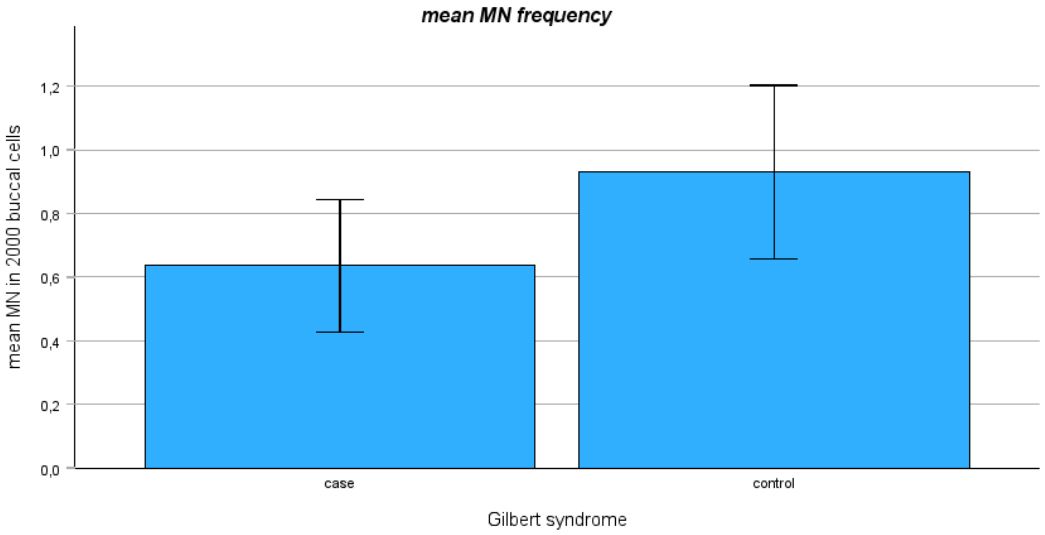

Figure 11: Bar chart showing the MN frequency compared between the Gilbert syndrome case and control group. Error bars show the 95% confidence interval. Mean MN in case group: 0.636 (± 0.209); Mean MN in control group: 0.930 (± 0.272); MN cells were counted in 2000 countable buccal cells. MN= micronucelated cell

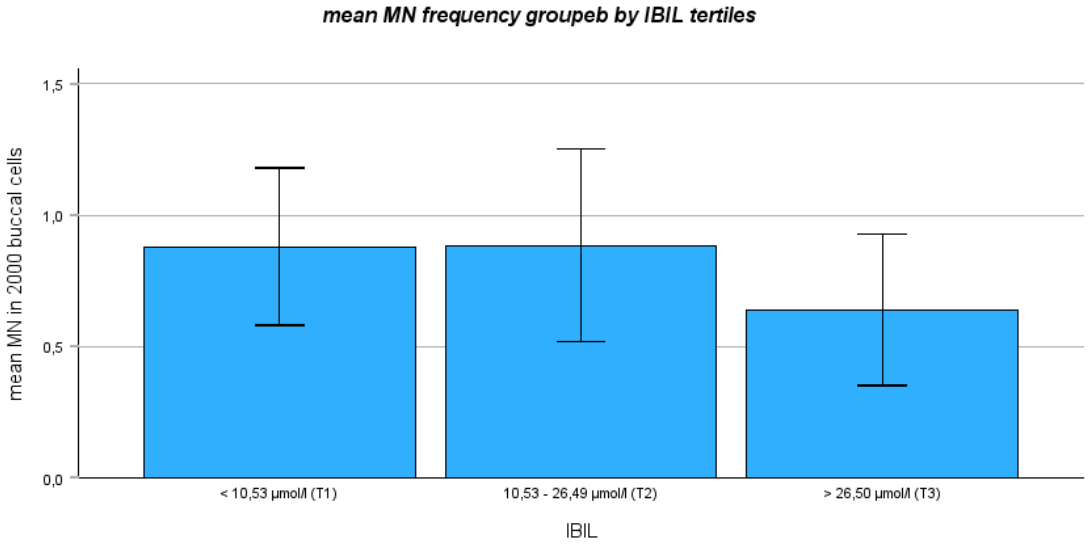

Figure 12: Bar chart showing the mean MN frequency compared between IBIL tertiles (T). MN cells were counted in 2000 buccal cells. Error bars show the 95% confidence interval. Mean MN in T1: 0.880 (±0.300), mean MN in T2: 0.885 (±0.367), mean MN in T3: 0.640 (±0.289); MN = micronucleated cell; IBIL = indirect bilirubin (unconjugated bilirubin)

The blood level of unconjugated Bilirubin (IBIL) was taken from subjects at baselevel before the activity challenge. When looking at those baseline IBIL levels but divided into tertiles, a negative correlation between the bilirubin concentrations and the MN frequency becomes apparent. However, this correlation could not be proven to be significant. There is also no significant difference between tertile 1 (<10,53 µmol/l) and tertile 3 (>26,50 µmol/l). (figure 12)

MN cells are one possibility to measure the extent of underlying DNA damage caused by external or internal factors such as elevated bilirubin in the serum. Although MN are the most extensively investigated chromosomal instability parameter in cells, other types of chromosomal cell damage can also be relevant. The following table shows an overview of different cell types that were counted for this study. It shows the mean values with their standard deviations and compares between case and control group. The most relevant cells regarding DNA damage besides MN cells are CC, BN, NBUD and BE cells. [Bolognesi et al. 2013]

	Gilbert syndrome				
	YES		**NO**		
	Mean	SD	Mean	SD	p-value
MN	0.64	± 0.21	0.93	± 0.27	0.135
DC	803.50	± 20.69	829.23	± 17.33	0.067
BC	14.98	± 2.48	13.72	± 1.91	0.432
CC	1.23	± 0.27	1.70	± 0.32	0.030*
KL	86.36	± 12.07	76.02	± 13.76	0.162
KR	87.34	± 14.31	71.79	± 10.22	0.177
P	0.73	± 0.22	0.79	± 0.27	0.876
BN	2.45	± 0.44	2.77	± 0.60	0.408
NBUD	0.36	± 0.17	0.56	± 0.24	0.280
BE	2.77	± 0.87	2.70	± 0.80	0.727

Table 4: Chromosomal instability parameters in case and control group. Mean values ± standard deviation and p-values of all the different cell types in buccal cells compared between the Gilbert syndrome case group and control group. MN counted in 2000 buccal cells and all other cell types in 1000 buccal cells. Only the difference between the CC cells is significant between case and control group ($p < 0.05$). MN - micronucleated cell; DC - differentiated cell; BC - basal cell; CC - condensed chromatin; KL - karyolitic cell; KR - karyorrhectic cell; P - pycnotic cell; BN - binucleated cell; NBUD - nuclear Bud; BE - broken egg

When looking at figure 13, participants with GS show slightly reduced mean chromosomal damage parameters compared to the controls. When testing for significance only the difference in CC frequency is significant (p = 0.030) between individuals displaying GS and the control group. For BE, NBUD and BN frequency there is no significant difference between the groups. Although it could be argued that a tendency can be seen even though it is still not significant.

Despite only CC cells showing a significant difference between case and control group, a tendency of slightly less chromosomal damage in Individuals with GS can be seen throughout the results. This correlates with the expected outcome because of the antioxidant effects of slightly more bilirubin in the blood and similar studies that have been done in the past. [Wallner et al. 2012]

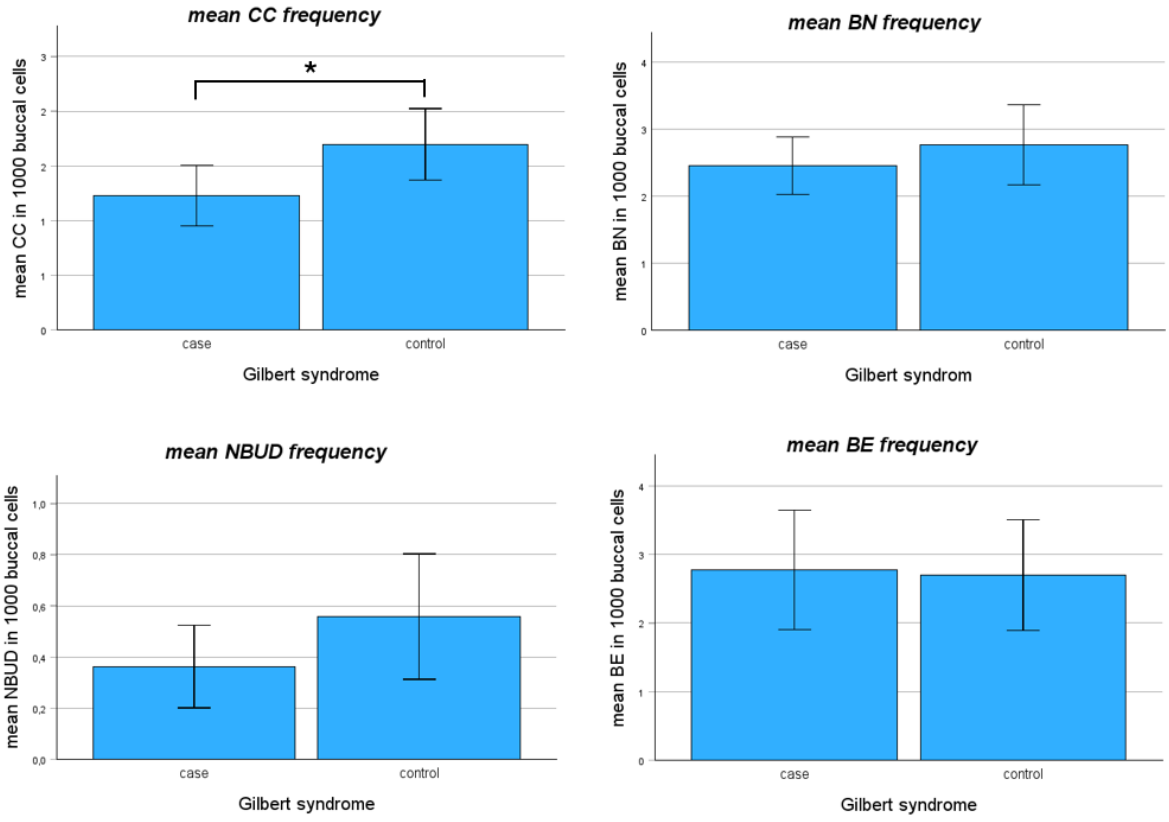

Figure 13: Bar charts showing frequencies of different cell types counted in buccal cells indicating DNA damage compared between the Gilbert syndrome case and control group. Error bars show the 95% confidence interval. Mean CC count in case group vs. control group: 1.23 (±0.27) vs. 1.70 (±0.32); mean BN count in case group vs. control group: 2.45 (±0.44) vs. 2.77 (±0.60); mean NBUD count in case group vs. control group: 0.36 (±0.17) vs. 0.56 (±0.24); mean BE count in case group vs. control group: 2.77 (±0.87) vs. 2.70 (±0.80); Cells were being counted in 1000 countable buccal cells. CC - condensed chromatin; BN - binucleated cells; NBUD - nuclear Bud; BE - broken egg

4.2. Factors that might influence chromosomal instability

It is already known that aging has a major impact on oxidative stress in the human body. Various studies showed a significantly positive correlation between older age and a higher MN frequency in lymphocytes. [Fenech M., Bonassi S. 2011] However, there are only a few studies that investigated the effect of chromosomal damage in buccal cells. Some studies showed significantly higher MN frequency in older subgroups whereas other only show tendencies or sometimes even no effect of age on buccal cells at all. [Hopf et al. 2020]

In the study population of the BiliMetHealth study, age had a slight impact on MN frequency in buccal cells. The MN count only slightly positively correlates with age. Age alone does not have a significant influence on the MN count in our data (p = 0.651). (figure 14)

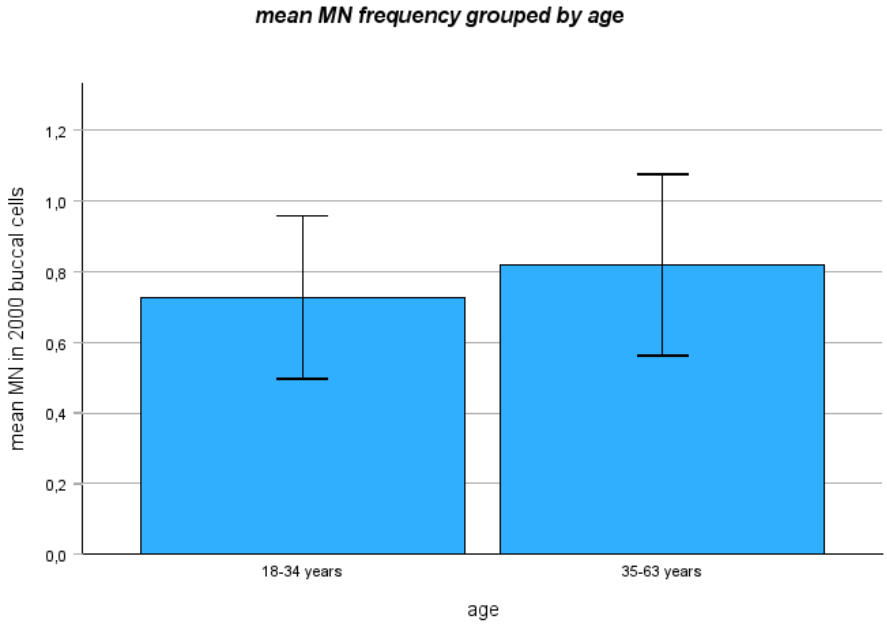

Figure 14: Bar chart showing the mean MN frequency grouped by age. MN cells were counted in 2000 countable buccal cells. Error bars show the 95% confidence interval. Mean MN count 18-34 years = 0.73 (±0.23); mean MN count 35-65 = 0.82 (±0.25); MN = micronucleated cells

As can be seen in figure 15, the difference in MN frequency between case and control group, grouped into a younger and an older age group is depicted. For these purposes a cutoff-point of 35 years was set to split the data evenly into a “younger” group from 18-34 years and an “older” group from 35-63 years old. Mean MN count in the case group in individuals from 18-34 years is 0.62 (±0.30) whereas it is 0.65 (±0.31) in individuals in the case group from 35-63 years. However, in the control group a higher mean MN count can be seen in the older age group compared to the younger age group. In the control group the mean MN frequency is higher when comparing the younger group (1.05±0.44 MN) to the older controls (0.83±0.36 MN). When comparing the MN count in the control groups grouped by age (younger = 18-34, older = 35-63) no significant difference (p = 0.389) between the two age groups could be found.

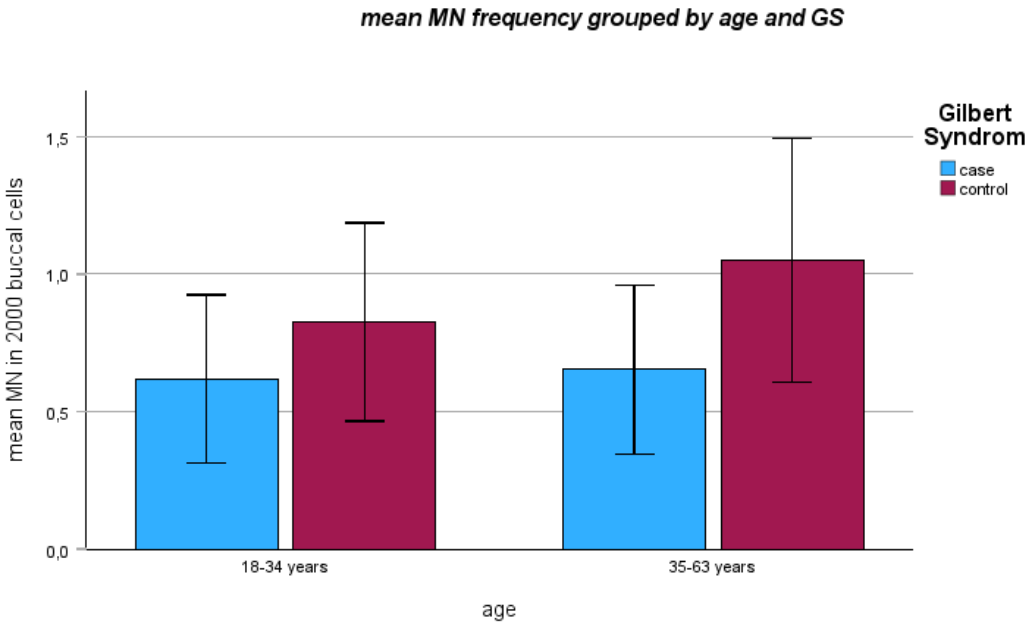

Figure 15: Grouped bar chart showing mean MN frequency in different age groups comparing Gilbert syndrome case and control group. Mean MN 18-34 case: 0.62 (±0.30), mean MN 18-34 control: 0.83 (±0.36), mean MN 35-63 case: 0.65 (±0.31), mean MN 35-64 control: 1.05 (±0.44); MN frequency was counted in 2000 countable buccal cells. MN - miconucelated cell

Although the difference between MN count in the older GS group compared to the older non-GS group is not significant, a tendency can be seen. These types of tendencies towards less elevated means in MN frequency in older subjects with GS can also be found in other publications. This indicates antioxidant abilities of bilirubin that might be protective in buccal cells, specifically in MN formation during aging. [Wallner et al. 2012]

When comparing MN frequencies between the age groups but grouped into IBIL tertiles instead of GS groups, those tendencies of fewer MN cells in the older population with the most bilirubin in the blood can also be seen. However, it is also interesting to note, that the tertile with the highest bilirubin concentrations in the blood also has the lowest number of MN cells counted, regardless of the age. No significant differences could be found between any of the groups in figure 16. (figure 16)

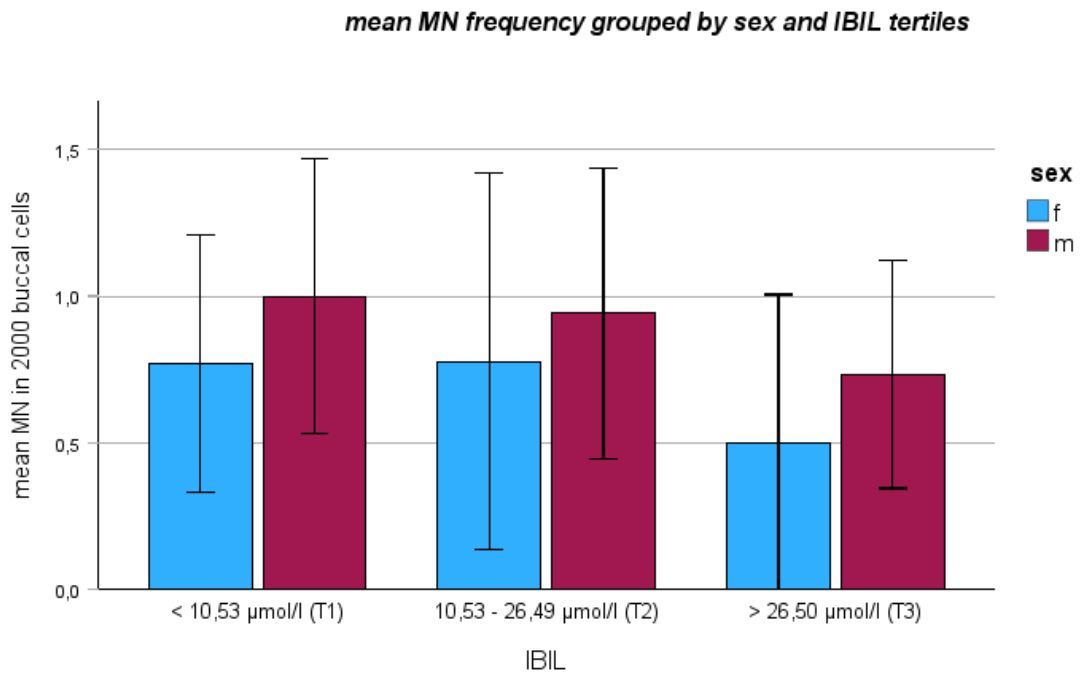

Figure 16: Grouped bar chart showing mean MN frequencies in different age groups compared between IBIL tertiles. MN frequency was counted in 2000 countable buccal cells. Error bars show the 95% confidence interval. Mean MN T1, f: 0.77 (±0.44), mean MN T1, m: 1.00 (±0.47), mean MN T2, f: 0.78 (±0.64), mean MN T2, m: 0.94 (±0.50), mean MN T3, f: 0.50 (±0.51), mean MN T3, m: 0.73 (±0.39); MN = micronucleated cell; IBIL = indirect bilirubin (unconjugated bilirubin)

When comparing the MN count between men and women this study population shows a slightly higher mean MN count in men compared to women. Males of this study population have a mean MN frequency of 0.87 (±0.23) whereas women have a mean MN frequency of 0.64 (±0.24) MN cells counted. This difference between the MN counts is not significant. (figure 17)

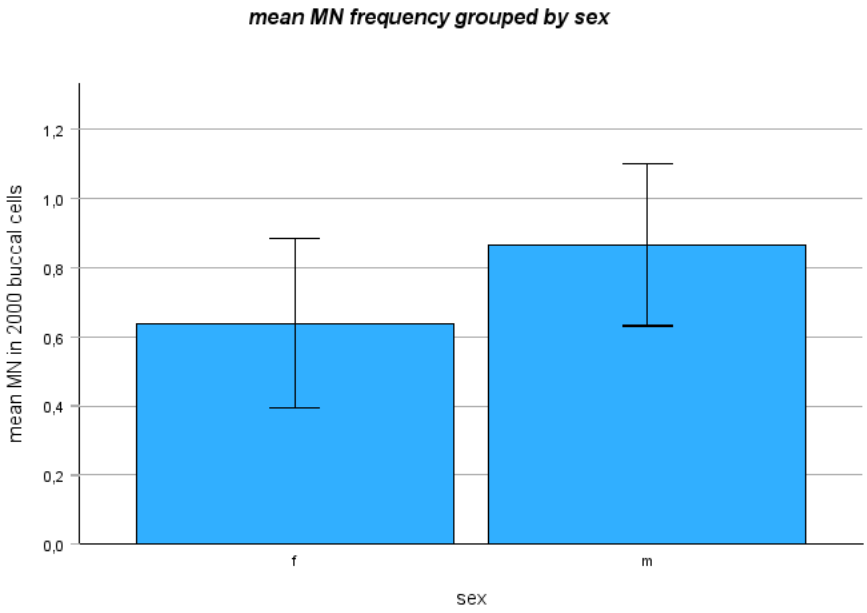

Figure 17: Bar chart showing mean MN frequency grouped by sex. MN frequency was counted in 2000 buccal cells. Error bars show the 95% confidence interval. Mean MN f: 0.64 (±0.24), mean MN m: 0.87 (±0.23); MN = micronucleated cell; f = female; m = male

When comparing MN frequency not only grouped by sex but additionally also into GS groups, the data shows a similar mean MN frequency between men and women comparing only the case group. It also shows a similar MN frequency between women in the case group (0.61±0.39) and women in the control group (0.67±0.34). However, a trend of more MN

counted in men of the control group (1.12±0.40) than women of the control group (0.67±0.34) as well as men of the control group (1.12±0.40) and men of the case group (0.65±0.26) can be seen. (figure 18) This difference is not significant (p = 0.132) and correcting for sex does also not further explain the data. This shows an interesting development in comparison to the literature, where most studies report a higher MN count in women than in men in PBMCs. [Fenech M., Bonassi S. 2011] However, the majority of studies on buccal cells report no effect of sex on MN count. [Hopf et al. 2020] [Fenech et al. 2011]

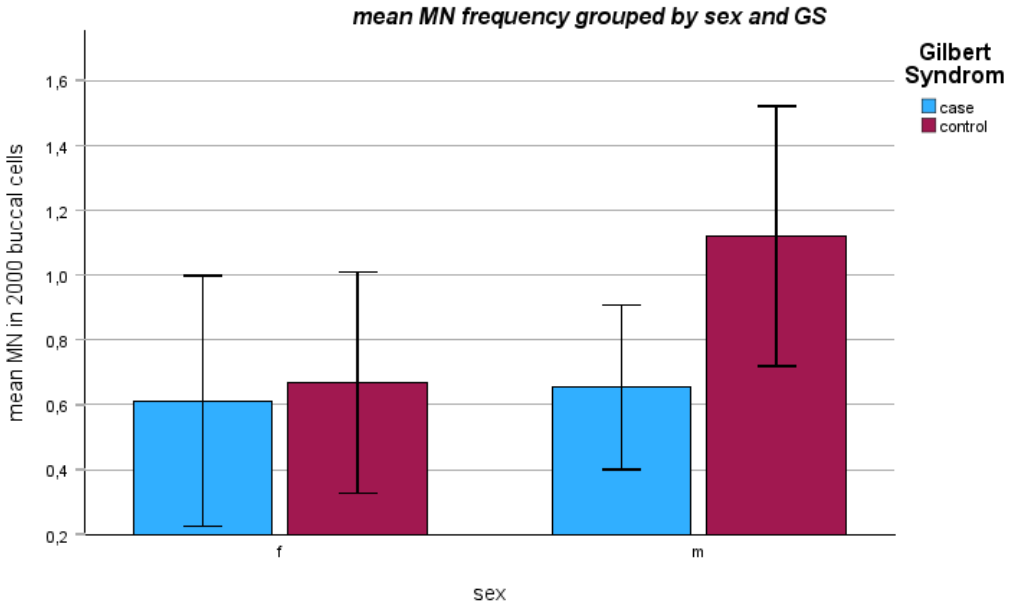

Figure 18: Grouped bar chart showing MN frequency grouped by Gilbert sydrome and sex. Error bars show the 95% confidence interval. Mean MN count in w, case: 0.61 (±0.39); mean MN count in m, case: 0.65 (±0.26); mean MN count in w, control: 0.67 (±0.34); mean MN in m, control: 1.12 (±0.40); MN were counted in 2000 buccal cells; MN - miconucleated cell; w – women; m - men

5. Discussion

Gilbert syndrome (GS), which can also be called benign, unconjugated hyperbilirubinemia, is a condition that leads to an elevated level of unconjugated bilirubin in the blood. [Watson, Golla n. 1989] Although GS is not the only reason for a slightly elevated bilirubin level in the blood, an average prevalence of 5-10% of the European population makes it an important condition that should be investigated thoroughly. [Wagner et al. 2018]

Some studies suggest that a slightly elevated bilirubin level, as is the case in individuals with GS, exerts a protective role against non-communicable diseases (NCDs). Those diseases include various forms of cancer, respiratory diseases, NAFLD, T2DM and cardiovascular diseases. [Horsfall et al. 2011] [Weaver et al. 2018] A recent analysis of a cohort study found beneficial effects of mild hyperbilirubinemia on multiple metabolic health parameters. They found that slightly elevated unconjugated bilirubin concentrations are associated with lower BMI, triglyceride, cholesterol, CRP and insulin in the blood as well as higher HDL-cholesterol, glutathione and creatinine in comparison to individuals with lower bilirubin concentrations. The authors of this analysis conclude that elevated bilirubin concentration is a protective factor against cell and DNA damage and therefore affect cellular changes associated with aging. [Schoissengeier et al. 2024] This coincides with other studies that suggest that a mild hyperbilirubinemia is associated with protection from all-cause mortality maybe also through its protection from DNA and chromosomal damages. [Wagner et al. 2015]

The underlying physiological reasons for these protective mechanisms include antioxidant properties of bilirubin as well as the whole “bilirubinomics” system. “Bilirubinomics” is a term for various effects of bilirubin in multiple mechanisms in the human body. Those mechanisms where bilirubin plays a role include the modulation of various cellular pathways, the activation of receptors and protein phosphorylation. The field of the so called “bilirubinomics” investigating all the possible effects of bilirubin on the human body and its cellular pathways is relatively new. [Vitek, Tiribelli. 2021] [Gazzin et al. 2016]

The mechanisms of the antioxidant effect of bilirubin on the other hand are very well known and studied. Reactive oxygen species (ROS) can exert damaging effects on all types of cells in

the body and lead to DNA damage and therefore chromosomal instability. The antioxidant properties of bilirubin are hypothesized to protect cells from chromosomal damage and diseases as a consequence thereof. [Wallner et al. 2012] [Bulmer et al. 2008] Bilirubin exerts its antioxidant properties by protecting the cell interior against harmful stimuli. [Vitek L. 2020] Studies report, that bilirubin plays an indispensable cytoprotective role against cell damage by observing that stress induced cell damage was ameliorated by the addition of bilirubin. [Takeda et al. 2015] This antioxidant mechanism of bilirubin works by neutralizing ROS through cells having bilirubin recovery pathways to maintain their antioxidant homeostasis by transforming heme into biliverdin and bilirubin again. Transforming heme into biliverdin and reducing biliverdin to bilirubin has antioxidant and anti-inflammatory effects. [Conti C. 2021]

One tissue that is particularly susceptible to ROS and DNA damage is the buccal mucosa, as it is primary point of contact between the body's tissue and external factors such as food, ingredients and environmental microbes. Because more than 90% of tumours derive from epithelial cells the buccal mucosa may in theory even be a better predictor for human biomonitoring than for example lymphocytes. [Cairns J. 1975] Most studies investigated DNA damage in lymphocytes and many of them found a link between increased MN frequency in lymphocytes and diseases like cancer. [Chang et al. 2010] Recent studies investigating both lymphocytes and buccal cells suggest a positive correlation in both surrogate tissues concerning chromosomal damage like MN. [Bonassi et al. 2011] [Sommer et al. 2020]

Before the standardised BMCyt was introduced, studies investigating chromosomal instability parameters in buccal cells varied a lot in epidemiology and statistics. One drawback of the method was the influence of the different staining methods on chromosomal damage in buccal cells as well as the effect of interlaboratory differences. Therefore, results from studies that have not used the standardized protocol by Thomas et al. 2009 can hardly be compared when it comes to interpretation of the results. [Bonassi et al. 2011] [Ceppi et al. 2010] The BMCyt method introduced a standardised method for sampling, staining and scoring of buccal cells, with minimum impact on chromosomal structures. The BMCyt is a well-established method for estimation of chromosomal damage in form of MN formation in buccal cells and was used to obtain the results for this thesis. [Thomas et al. 2009]

A study from 2014 showed that MN frequency and various chromosomal instability parameters in buccal cells are also valid markers for biomonitoring human cancer risk. [Flores-Garcia et al. 2014] Chromosomal damage in buccal cells is well documented to be influenced by age, gender and lifestyle choices like smoking. [Upadhyay et al. 2019] On the other hand, a lot of studies could not confirm the influence of aging and lifestyle choices on buccal cell chromosomal damage and suggest that the method has its limitations. [Ceppi et al. 2010] [Rickes et al. 2010] The authors of Ceppi et al. 2010 for example suggest raising the number of buccal cells counted from 1000-2000 to 4000. By increasing the number of buccal cells counted to 4000 the width of the confidence interval would decrease which would therefore reduce the variability of the results. This would lead to more precise estimates and more precise results with a smaller width of the confidence interval. [Ceppi et al. 2010] One other limitation is the lower repair capacity and higher cell turnover rate of buccal cells compared to other cells, which might be one explanation why results often differ compared to other cell types. [Wultsch et al. 2013]

This thesis looked at MN specifically as a parameter to investigate the effects of unconjugated bilirubin concentrations on chromosomal damage in buccal cells using the BMCyt. The samples for this study were obtained as part of the Bilirubin Metabolic Health study (BiliMetHealth). BiliMetHealth is a case control study carried out at the University of Vienna within an FWF project. A total of 92 subjects were enrolled in the study with the case group having GS and the control group being age and gender matched. Comparing MN frequencies between case and control group, no significant difference could be found between the groups in this study. No significant age effect could be found for buccal MN frequency either. When looking at other forms of chromosomal damages, only CC cells showed a significantly lower frequency in the case group compared to the control group. Comparing age groups shows the highest MN count in the control group above 35 years compared to the case group or younger subjects. Although this difference in mean MN count is not significant, this indicates a possible protective effect of bilirubin in the aging process.

When comparing mean MN count between men and women, more MN were counted in the buccal cells of men compared to women, but no significant difference was found. This

coincides with studies on buccal cells, which report no significant effect of sex on MN frequency. [Hopf et al. 2020] [Fenech et al. 2011] However, studies on PBMCs clearly show a higher MN count in women than in men. This shows an interesting development in comparison to our data, where a tendency of more MN in buccal cells of men compared to women was found. [Fenech M., Bonassi S. 2011]

In summary, it can be said that aging as well as all kinds of different diseases coincide with cellular changes like chromosomal damage. Bilirubin can protect cells from chromosomal damage by exerting its antioxidant properties and protecting the cell interior against harmful stimuli. Data from the last decades convincingly demonstrates a protective effect of mildly elevated serum bilirubin concentrations against various diseases and show a positive effect of bilirubin on cellular changes associated with aging. The BMCyt makes it possible to use buccal cells to investigate chromosomal damage and therefore to examine the effects of bilirubin on chromosomal damage in buccal cells.

6. Conclusion

This study aimed to investigate the influence of a slightly elevated unconjugated bilirubin level in the blood due to GS on chromosomal damage parameters in buccal cells. As a method for investigating buccal cells, the BMCyt was used. This study mainly concentrated on MN frequency as a chromosomal damage parameter in buccal cells.

The theory behind the hypothesis of this study suggests a protective and antioxidant effect of bilirubin. [Bulmer et al. 2008] Not only the antioxidant effect of bilirubin has a positive influence on chromosomal damage parameters in buccal cells. Other positive influences include the modulation of various cellular pathways, the activation of receptors and protein phosphorylation. [Vitek, Tiribelli. 2021] [Gazzin et al. 2016] Therefore, less chromosomal damage in buccal cells is expected in individuals having GS compared to individuals not having GS.

Comparing MN frequencies between case and control group, no significant difference could be found between the groups in this study. No age effect could be found for buccal MN frequency either. When looking at other forms of chromosomal damages, only CC cells showed a significantly lower frequency in the case group compared to the control group. Although no significant differences between the groups could be found, a tendency of less chromosomal damage in the case group can be seen when looking at MN frequency as well as CC, BN and NBUD frequencies. Comparing age groups shows the highest MN count in the control group above 35 years compared to the case group or younger subjects. Although this difference in mean MN count is not significant, this indicates a possible protective effect of bilirubin in the aging process.

Not many studies have been published looking at the effects of GS on buccal cell chromosomal damage parameters so far. The ones published coincide with the results of this study. They also show similar tendencies of less chromosomal damage in buccal cells in individuals with GS. Taking together findings of this and other similar studies, the findings suggest that chromosomal damage is decreased in older subjects with GS. This indicates that individuals with GS might be protected against variations of genetic material when aging. This leads to the

conclusion that unconjugated bilirubin has a protective effect on chromosomal stability. [Wallner et al. 2012] However, more and larger studies need to be done on this topic to fully understand the impact of unconjugated bilirubin on chromosomal damage in buccal cells, especially in combination with aging.

Summary

Many studies investigating Gilbert syndrome (GS) conclude that a slightly elevated unconjugated bilirubin level in the blood represents a protective factor for multiple endpoints including cardiovascular diseases, various forms of cancers and diabetes mellitus type 2. [Strassburg C. 2010] Some studies even suggest lower all case mortality or propose GS to be an evolutionary advantage. [Strassburg et al. 2008] [Wagner et al. 2015] Not only does GS protect from diseases but its protective properties can also be seen when investigating chromosomal stability parameters in different tissues of the human body. One of those tissues is the buccal mucosa, which is an easily accessible tissue for sampling. In buccal cells, the regenerative capacity of tissues as well as the propensity for cell death and DNA damage can be investigated. One way to investigate chromosomal instability, which is mainly caused by DNA damage, is to examine the micronucleus (MN) frequency in buccal cells. Events like regenerative capacity or micronucleus frequency in buccal cells provide information about the health condition of the cells within a specific tissue and the conditions can be considered as indicators or parameters for healthy aging in general. [Thomas et al. 2009] This thesis investigated the properties that benign, unconjugated hyperbilirubinemia has on chromosomal instability parameters, especially MN formation, in buccal cells by using the buccal micronucleus cytome assay (BMCyt). The BMCyt is a well-established method for estimation of chromosomal damage in form of MN formation in buccal cells. [Thomas et al. 2009] The samples for this study were obtained as part of the Bilirubin Metabolic Health study (BiliMetHealth). BiliMetHealth is a case control study carried out at the University of Vienna within an FWF project. A total of 92 subjects were enrolled in the study, with the case group having GS and the control group being age and gender matched. The results showed no significant difference in MN count between the case and control group. No significant effect of sex or age on MN in buccal cells could be found either. This coincides with the majority of studies on buccal cells, which report no significant effect of sex or age on MN frequency. [Fenech et al. 2011] However, a tendency of less MN in the case group was found as well as less MN in the older subgroup having GS compared to the older subgroup not having GS. Taking together findings of this and other similar studies, the findings suggest that chromosomal damage is decreased in older subjects with GS. This indicates that individuals with GS might

be protected against variations of genetic material when aging, which leads to the conclusion that unconjugated bilirubin has a protective effect on chromosomal stability. [Wallner et al. 2012] However, more and larger studies need to be performed to determine the ability of buccal cells to accurately depict the influence of GS on chromosomal damage parameters.

Zusammenfassung

Gilbert Syndrom ist durch einen leicht erhöhten Bilirubin Spiegel im Blut detektierbar und ist auch als gutartige, unkonjugierte Hyperbilirubinämie bekannt. [Sanyal et al. 2018] Zahlreiche Studien belegen positive Effekte eines leicht erhöhten unkonjugierten Bilirubinspiegels im Blut auf verschiedene Biomarker. Diese positiven Effekte umfassen das geringere Risiko für Kardiovaskuläre Krankheiten, Diabetes Mellitus Typ 2 sowie mehrere verschiedene Krebsarten und in einigen Studien wird auch von einer erniedrigten Gesamtmortalitätsrate berichtet. [Strassburg C. 2010] [Wagner et al. 2015] Nicht nur auf bestimmte Erkrankungen kann sich die Manifestation des Gilbert Syndroms (GS) positiv auswirken. Die positiven Effekte von GS können sich auch in verschiedenen Geweben widerspiegeln wie zum Beispiel im Gewebe der Mundschleimhaut. Mundschleimhautzellen eignen sich sehr gut, um das regenerative Potenzial von Zellen zu untersucht und auch Marker für chromosomale Instabilität und DNA-Schäden der Zellen können in diesem Gewebe gemessen werden. Um DNA-Schäden der Mundschleimhautzellen zu untersuchen kann unter anderem die Frequenz der Mikrokerne untersucht und ausgewertet werden. Die Anzahl der Mikrokerne gibt Aufschluss über den Zustand der Mundschleimhautzellen und somit auch über den Zustand anderer Gewebe des Körpers. Mit dem sogenannten Buccal Micronucleus Cytome Assay (BMCyt) können Mundschleimhautzellen abgenommen, gefärbt und anschließend auf die genannten Zellschäden untersucht werden. [Thomas et al. 2009] Das Ziel dieser Masterarbeit war zu untersuchen, ob GS positive Effekte auf Parameter für chromosomale Schäden, insbesondere die Entstehung von Mikrokernen (MN), in Mundschleimhautzellen hat. Insgesamt wurden 92 Mundschleimhaut-Proben im Zuge der “Bilirubin Metabolic Health” (BiliMetHealth) Studie abgenommen und untersucht. Es konnte kein signifikanter Unterschied zwischen MN-Anzahl in der GS-Gruppe im Vergleich zur Kontrollgruppe festgestellt werden. Ebenso haben weder Geschlecht noch Alter eine Auswirkung auf die MN-Anzahl in Mundschleimhautzellen gehabt. Diese Ergebnisse stimmen mit denen aus der Mehrzahl an Studien überein, die an Mundschleimhautzellen vorgenommen wurde, bei denen ebenfalls kein Effekt von Alter oder Geschlecht auf MN-Anzahl festgestellt werden konnte. [Fenech et al. 2011] Es wurde jedoch eine Tendenz zu weniger MN in der Fallgruppe festgestellt, ebenso wie weniger MN in der älteren Untergruppe mit GS im Vergleich zur älteren Untergruppe ohne GS.

Zusammengenommen deuten die Ergebnisse dieser und anderer ähnlicher Studien darauf hin, dass Chromosomenschäden bei älteren Personen mit GS seltener vorkommen. Dies deutet darauf hin, dass Personen mit GS im Alter möglicherweise vor Variationen des genetischen Materials geschützt sind, was zu der Schlussfolgerung führt, dass unkonjugiertes Bilirubin eine schützende Wirkung auf die Chromosomenstabilität hat. [Wallner et al. 2012] Es müssen jedoch weitere und umfangreichere Studien durchgeführt werden, um herauszufinden, inwiefern Parameter chromosomaler Schäden in Mundschleimhautzellen den Einfluss von GS auf Zellschäden darstellen.

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