universität wien

MASTERARBEIT

Titel der Masterarbeit

„Assessment of DNA damage in females with type 2 diabetes mellitus“

verfasst von

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angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2015

Studienkennzahl lt. Studienblatt:	A 066 838
Studienrichtung lt. Studienblatt:	Lebensmittelqualität und -sicherheit
Betreut von:	Univ. Prof. Dr. Karl-Heinz Wagner

Meiner lieben Familie

Zwei Dinge sind zu unserer Arbeit nötig: Unermüdliche Ausdauer und die Bereitschaft, etwas, in das man viel Zeit und Arbeit gesteckt hat, wieder wegzuwerfen.

Albert Einstein

Danksagung

Zuerst möchte ich mich recht herzlich bei Univ. Prof. Dr. Karl-Heinz Wagner für die Übernahme des Masterarbeitsthemas bedanken. Vielen Dank für die nette Betreuung und die stetige Hilfe.

Ein großes Dankeschön geht an Dipl. troph. Annemarie Grindel. Vielen Dank für großartige Betreuung und Unterstützung im Labor und beim Schreiben der Arbeit. Du warst mir eine große Hilfe.

Ich möchte mich bei dem ganzen Team der "MIKRODIAB" Studie bedanken für die gute Zusammenarbeit. Besonders bedanken möchte ich mich bei Alex und Amelie.

Ich bedanke mich auch bei meinem Freund Oliver, für die Unterstützung und, dass du immer ein offenes Ohr für mich hattest.

Der größte Dank geht an meine Familie. Ich danke meinen Eltern für die Unterstützung und Ermöglichung des Studiums. Meiner Schwester Nicole danke ich für alles. Du warst meine größte Stütze in jeder Minute. Vielen Dank dafür, dass du immer die richtigen Worte gefunden hast und immer für mich da warst.

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List of Abbreviations

AGEs	advancedglycation end products
AP	apurinic/apyrimidinic
CAT	catalase
DM	diabetes mellitus
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
Endo III	endonuclease III
FBS	fetal bovine serum
FFAs	free fatty acids
FPG	formamidopyrimidine DNA glycosylase
GDP	gross domestic product
GSH	glutathione
GSSG	oxidised glutathione
GPx	glutathione peroxidase
HbA1c	hemoglobin A1c
H_2O_2	hydrogen peroxide
H_2O	water
IDF	International Diabetes Federation
IDDM	insulin dependent diabetes mellitus
IGT	impaired glucose tolerance
IGF	impaired fasting glucose
LMA	low melting agarose
MetS	metabolic syndrome
NADPH	nicotinamide adenine dinucleotide phosphate
NIDDM	non insulin dependent diabetes mellitus
NMA	normal melting agarose
NOX	NADPH oxidize
NYHA	New York Heart Association

OGTT	oral glucose intolerance test
OGG1	oxoGua-DNA glycosylase
PBMC'S	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PKC	protein kinase C
ROS	reactive oxygen species
SCGE	Single Cell Gel Electrophoresis
SOD	superoxide dismutase
SU	sulfonylurea
T2DM	Type 2 Diabetes Mellitus
T1DM	Type 1 Diabetes Mellitus
8-oxoGua or 8-OHdG	8-Oxoguanine (= 8-Hydroxydesoxyguanosin or8-oxo-7,8-dihydroguanine)

1 Introduction

Worldwide 387 million people are suffering from diabetes mellitus (DM), from which Type 2 diabetes mellitus (T2DM) is representing approximately 90-95 % of all cases (ADA, 2014; IDF, 2014a). By 2035, this number will rise to 592 million cases**.** This makes DM one of the fastest increasing diseases worldwide. The high DM rate is not just a phenomenon of industrialized countries, but also of developing countries. The highest prevalence is noticed in low- and middle-income countries. In Austria the prevalence of DM was 9 %, in 2014 (IDF, 2014a). Risk factors such as aging, unhealthy diet, physical inactivity and obesity, are mainly contributing to the increase in T2DM (Giovannucci et al., 2010). DM is characterized by an increase in the blood sugar levels, the so-called hyperglycemia, which is the result of impaired insulin secretion and/or insulin action (ADA, 2003)**.** Especially in T2DM, chronic hyperglycemia, and its common accompanied risk factors hypertension and dyslipidemia, are leading to a direct toxic effect of sugar on tissues, and thereupon to changes in small and large vessels, called micro- and macrovascular complications (Stamler et al., 1993)**.** Metabolic factors of the disease can cause damage to DNA, what makes DM a condition linked to DNA damage. Oxidative stress is probably the main agent for DNA damage in diabetics (Lee and Chan, 2015). In patients with poor glycaemic control DNA damage is considered to be higher (Xavier et al., 2015). In addition, mutations which are playing key roles in the carcinogenesis can be caused by DNA damage (Lee and Chan, 2015). Consequently DM is associated with an increased risk for cancer worldwide, except prostate cancer (Giacco and Brownlee, 2010). Pathophysiological factors of DM can cause DNA damage, and DNA damage in turn can cause mutations, and these mutations are linked to carcinogenesis. For assessing DNA damage the comet assay has become one of the standard methods in research (Collins, 2004).

The first aim of the present master's thesis, which was performed within the cross-sectional study "MIKRODIAB", was to examine the relationship between HbA1c and DNA damage in females with T2DM. Secondary aim was to evaluate whether the duration of T2DM is linked to DNA damage.

2 Literature survey

2.1 Definition and classification of Diabetes Mellitus

The term DM refers to a group of metabolic disorders (ADA, 2003). These metabolic disorders have in common an increase in blood sugar levels, which leads to chronic hyperglycemia (Brownlee, 2001). Hyperglycemia results from an impaired insulin secretion and/or insulin action. Chronic hyperglycemia leads to severe disturbances of secretion and/or effects of insulin (ADA, 2003). Therefore it is associated with long-term complications and functional disorders or failure of organs or tissues (eyes, kidneys, heart, nerves and blood vessels), the so called micro and macrovascular complications (Brownlee, 2001; UKPDS, 1998). A severe hyperglycemia runs from classical symptoms such as polydipsia, polyuria, unexplained weight loss, increased susceptibility for infections and serious visual problems to ketoacidosis or non-ketoacidosis hyperosmolar syndrome (ADA, 2003). DM is classified into four groups: type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), gestational diabetes mellitus (GDM), and other specific types of DM (table 1). In terms of T2DM initially, there is the early stage of DM, the so called pre-diabetes, which is designated by an abnormal glucose metabolism, but the criteria for the term DM are not completely fulfilled (Smushkin and Vella, 2010). Specific criteria were defined for the diagnosis of DM, such as fasting plasma glucose ≥ 126 mg/dl (≥7.0 mmol/l) (table 2). Therefore the oral glucose intolerance test (OGTT) or the fasting plasma glucose have been used for DM diagnosis in the last decades (ADA, 2014). At that time the gold standard for diagnosis was the measurement of glucose in venous plasma. Hence a preanalytical treatment of blood is necessary, because glycolysis needs to be inhibited. However epidemiological investigations have shown, that the hemoglobin A1c (HbA1c) can be used for diagnosis of DM. The sensitivity of HbA1c > 6.5 % is strong enough for the diagnosis of DM and HbA1c < 5.7 % is strong enough to exclude DM (Kerner W, 2012). So HbA1c is a known common marker for chronic hyperglycemia, since it reflects the average of the blood glucose level over a period of 2-3 months. For patients with DM, HbA1c plays an important role to manage their disease. Furthermore this marker correlates mainly with microvascular complications (ADA, 2014).

Table 1: Classification of DM (ADA, 2014)

I. **Type 1 diabetes mellitus (T1DM):** Characterized due to beta cell destruction, which is usually leading to an absolute insulin deficiency. Which is mostly immune mediated or sometimes idiopathic.

   About 5-10 % are suffering from this form of DM.

II. **Type 2 diabetes mellitus (T2DM):** Characterized by a progressive insulin secretory defect on the basis of an insulin resistance.

   About 90-95 % are suffering from T2DM.

III. **Gestational diabetes mellitus (GDM):** GDM is a form of DM which is diagnosed in the second or the third trimester of a pregnancy, but which is not a clearly manifest DM.

IV. **Specific types of diabetes:** Due to many other causes, like:

- genetic defects of beta cell dysfunction or insulin action
- other genetic syndromes
- endocrinopathies
- diseases of the exocrine pancreas
- drug- or chemical-induced DM
- infections
- uncommon forms of auto immune-mediated DM

Table 2: Diagnosis criteria of DM (ADA, 2014)

- HbA1c ≥ 6.5 %
- a random plasma glucose ≥ 200 mg/dl (≥ 11.1 mmol/l)
- Fasting plasma glucose ≥ 126 mg/d (≥ 7.0 mmol/l)
- OGTT- two-hour plasma glucose ≥ 200 mg/dl (≥ 11.1 mmol/l)

2.1.1 Type 2 Diabetes mellitus (T2DM)

T2DM was previously designated as non–insulin dependent diabetes (NIDDM), or even adult-onset diabetes. T2DM includes individuals who have an insulin resistance and they usually have a relative than an absolute insulin deficiency (DeFronzo et al., 1979; Olefsky et al., 1982). At the beginning insulin treatment is not essential for patients and some individuals will never need any insulin treatment for daily life. Several causes can induce this type of DM. The specific etiologies are not explored yet (ADA, 2003). Most of patients suffering from T2DM are obese. A certain degree of insulin resistance can be induced by obesity it selves (Bogardus et al., 1985). Moreover, those ones who are not obese may have a very high percentage of total body fat, predominantly as android distribution (Kissebah et al., 1982). Most of the time T2DM remains undiagnosed in the first years, since hyperglycemia is developing stepwise. That means that in an early stage DM is difficult to diagnose (Fujimoto et al., 1987; Harris, 1989). The insulin secretion is not working properly in diabetics and is even insufficient to compensate for an insulin resistance. Pharmacological treatment and/or weight reduction is needed to improve insulin resistance (Firth et al., 1986; Henry et al., 1986; Wing et al., 1994). Furthermore T2DM patients are under high risk of developing micro and macrovascular complications (Fujimoto et al., 1987; UKPDS, 1998). For T2DM, many risk factors are reported, like increased age (Skyler and Oddo, 2002), ethnic subgroups (Egede and Dagogo-Jack, 2005) family history and lifestyle factors such as diet and physical inactivity (Schulz et al., 2006; Zimmet et al., 2001). The family history is a significant factor and genetic predisposition is supposed to be higher in T2DM than in the autoimmune form of T1DM (Barnett et al., 1981; Newman et al., 1987). Moreover, overweight or obesity is considered to be one of the strongest determinants for DM (Hu et al., 2001). There is also evidence, that active smoking is associated with a higher risk of T2DM (Willi et al., 2007). Furthermore some candidate genes for developing T2DM are described (Taneera et al., 2012). Another aspect is depression, since DM is associated with a higher risk of depression, compared to the general public. So depression can be seen as an own risk factor for DM (De Hert et al., 2009). Subjects which are at very high risk for developing T2DM are of course those ones which were suffering from pre-diabetes (IGT, IFG and HbA1c between 5.7 % and

6.4 %) and subjects with the metabolic syndrome (Griebler, 2013). A fact for consideration is that the quality of life is reduced in patients suffering from T2DM compared to healthy subjects of the same age (Golicki et al., 2015). One main solution approach to decrease T2DM is definitely a healthy lifestyle. This was shown in the U.S. where subjects at high risk for developing DM needed to undergo a program with lifestyle-modification. At least 150 minutes of physical exercise per week and 7 % of weight loss were the goals. This lifestyle-modification led to a reduction in the incidence for DM in subjects at high risk (Knowler et al., 2002). Therefore early screening especially in high risk subjects and lifestyle modifications could lead to a decrease in T2DM (Griebler, 2013). Other approaches are the use of a new generation of pharmaceuticals (Leahy, 2005).

2.1.1.1 Pathogenesis

DM is a metabolic disorder and as mentioned before characterized by an absolute or relative insulin deficiency (DeFronzo et al., 1979). At the starting point of T2DM there is a decrease in insulin deficiency with normal or increased insulin secretion, the so-called insulin resistance. A reason for the elevated insulin secretion may be an excessive intake of carbohydrates. Accompanied by decrease in insulin receptors, the so called down-regulation. The consequence is that there is a lack of glucose in the cells and at the same time there is a high level of glucose, circulating in the blood. Which in turn leads to an increase in insulin secretion in the beta cells of the pancreas (Elmadfa, 2009). But insulin resistance is not only a problem of a deficient uptake of glucose in the cells in response to the hormone insulin, but it is a complex syndrome that leads to risks for cardiovascular disease. There are numerous complex links between insulin resistance and dyslipidemia, hypercoagulability, hypertension and atherosclerosis (Ginsberg, 2000). If this condition of hyperglycaemia persists over a longer period, it will lead to an insufficiency of the beta cells and at least they will not produce enough insulin for the organism anymore (Elmadfa, 2009).

2.1.1.2 Complications

Especially in T2DM, hyperglycemia, and its common additional risk factors hypertension, and dyslipidemia, are leading to a direct toxic effect of sugar on tissues, and thereupon to changes in small and large vessels. The increase of blood glucose is primarily responsible for diseases of the eyes , kidneys and nerves. While dyslipidemia and hypertension can be attributed to complications of alterations in large vessels, that will lead to atherosclerosis and subsequently to heart attack and stroke (Stamler et al., 1993). Long-term complications of DM are versatile and include peripheral nephropathy, retinopathy, peripheral neuropathy and autonomic neuropathy. Peripheral nephropathy can lead to renal failure. Retinopathy can lead to loss of vision. Peripheral neuropathy can lead to diabetic foot ulcers and that may lead to amputations. And autonomic neuropathy can cause genitourinary, gastrointestinal, and cardiovascular symptoms and also sexual dysfunction (ADA, 2014). Diabetic neuropathy is the consequence of elevated blood glucose and blood pressure. Elevated blood glucose, together with high blood pressure and also high cholesterol are leading causes for retinopathy. Furthermore a cause of concern are pregnant women with DM, as they need to control and manage their circumstances carefully, because DM can lead to many severe complications during pregnancy and it has a negative effect on the fetus (IDF, 2014b). Hyperglycaemia increase the risk of traumatic pregnancies influenced by macrosomia (high birth weight) (Coustan and Imarah, 1984). Macrosomia can be further worsened by the excess levels of insulin, which is circulating in the placenta and that leads to a high birth weight of the newborn (Coustan, 2007). Moreover these babies often experience hypoglycaemia after birth (Metzger et al., 2008). In order to prevent the potential negative effects on the unborn child, women with DM have to achieve a target blood glucose level already before conception (IDF, 2014b). In the future these children are more likely to develop obesity and DM in life (Petitt et al., 1985). This also contributes to the rising number of diabetics worldwide (IDF, 2014b). Furthermore there is an increased risk of mortality from many types of cancer, except prostate cancer, among diabetics (Shikata et al., 2013). However the leading cause of death in diabetics is cardiovascular disease, mainly due to stroke and heart attacks (IDF, 2014b). Every seven seconds one person

dies as a consequence of DM (IDF, 2014a). Therefore maintaining blood pressure, cholesterol and predominantly blood glucose levels, at a normal level or even close to a level that is classified as normal is inevitable. Hence a regularly monitoring is needed for patients with DM. This may help to slow down or prevent complications of DM (IDF, 2014b).

2.1.2 Epidemiology

2.1.2.1 Epidemiology worldwide

Worldwide 387 million people are suffering from DM and it is one of the fastest increasing diseases all over the world. By 2035 this number will rise to 592 million cases**.** This huge number of cases leads to an incredible number of 612 billion US dollars, which were spent in health expenditure in the year of 2014. In the same year, 4.9 million deaths worldwide were caused by DM. All over the world, there is an increasing prevalence of T2DM. About 77 % of people who are suffering from DM are not living in high-income countries, but in low and middle-income countries. Most of the people with DM are within the age of 40 and 59. The largest number of DM is found in the west pacific, region with about 138 million people (IDF, 2014a). Urbanization is one alarming point. Due to urbanization there are worrying changes in lifestyle which is happening especially in developing countries. This transitions can lead to an increase in risk factors for non communicable diseases (Guariguata et al., 2014). In 2013, data from 381.8 million adults with DM, which were living in 219 countries and territories, was collected. Through this investigation, regional differences and variations in income groups were reported. In North America and the Caribbean region there was the highest regional prevalence (11 %, unadjusted). After age-adjustment the regions of Middle East and North Africa had the highest prevalence, with about 10.9 %. In Africa there will be the largest projected proportional increase (109 %) in DM cases in adults, by the year of 2035. The highest proportional increase in adults with DM will be expected in countries with low-income (108 %), followed by countries with lower middle-income (60 %), countries with upper middle-income (51 %), and finally countries with high-income (28 %) (Guariguata et al., 2014).

2.1.2.2 Epidemiology Austria

According to the data of the International Diabetes Federation the prevalence of DM in Austria was about 9.0 %, in 2014 (IDF, 2014c). This percentage includes diagnosed and undiagnosed cases. So there have been around 430,000 medically diagnosed diabetics (6 %) in Austria in the year of 2011, while the number of not-diagnosed diabetics was about 143,000 to 215,000 (2-3 %). This means that 573,000 to 645,000 people were suffering from DM in Austria, in the year of 2011**,** which are at increased risk of complications and late effects (Griebler, 2013). Moreover, in Austria the lifetime prevalence of DM rises with age. So 21.2 % suffered from DM with the age of 75 and older in 2006/07. Furthermore the prevalence is higher in the city of Vienna, Lower Austria and Styria compared to other regions of Austria, according to facts of 2006/07 (StatistikAustria;, 2007). These differences are associated with a healthier lifestyle and lower rates of obesity in Western Austria (Grossschadl and Stronegger, 2012). In contrast to other countries, in Austria there is no difference in the prevalence of DM concerning gender or migrant background (Griebler, 2013). But there is a difference in prevalence with regard to education, which was reported only in the case of women. More precisely, women with a higher education, had lower risk of developing obesity and DM (Kautzky-Willer et al., 2012). Furthermore the depression rate is higher in diabetics compared to non-diabetics. Obese women with DM have a higher prevalence of depression compared to obese men in Austria (Anderson et al., 2001). The high rate of diabetics and the constantly increasing number, especially of T2DM, are leading to an increase in diabetes-related complications and as well to diabetes-related mortality. In 2011, approximately 2,440 amputations (diabetic foot) were performed (Griebler, 2013), and according to mortality statistics, 3,121 people died due to of DM in 2014 (StatistikAustria;, 2014). However, documentation of deaths due to DM is incomplete in Austria, similar to other countries, and the real number of deaths is higher. All diabetes-related complications and of course medications are leading to a high health expenditure (Griebler, 2013). In 2012, 1.7 billion euro of health expenditure were used for patients with T2DM (SV/LEICON, 2012). The health expenditure amounted to 34,869 million euro or 10.8 % of the GDP, in 2013 (StatistikAustria;, 2013). Hence in Austria, a targeted lifestyle-modification and screening measures, like preventive

medical check-ups, are needed, in order to reduce the risk of developing T2DM (Griebler, 2013).

2.1.3 Diabetes mellitus a risk factor for cancer

Many studies have been conducted to evaluate the risk of cancer in association with DM, especially T2DM. Many studies show evidence for an increased risk of mortality from cancer among diabetics. This evidence has been demonstrated for indifferent types of cancer like liver, pancreas, breast, endometrium, colorectal, and kidney (De Bruijn et al., 2013; Giovannucci et al., 2010; Grote et al., 2010; McAuliffe and Christein, 2013; Shikata et al., 2013). In contrast, a decrease in the risk of prostate cancer in DM was observed (Harding et al., 2015). There are many potential risk factors, which were common to both diseases, DM and cancer. These risk factors are gender, aging, diet, physical inactivity, obesity, alcohol consumption, and also tobacco use. Some of them are modifiable, and some are not (Giovannucci et al., 2010). Several conditions may be involved in carcinogenesis including hyperglycemia, hyperinsulinemia, or chronic inflammation (Giovannucci, 2001; Giovannucci et al., 2010; Stattin et al., 2007). Many studies, which evaluate the association between DM and cancer, are conducted on large cohort groups. In Italy one large cohort of 167,621 subjects with DM were identified, and the follow-up mortality rate was observed. Results have shown that the mortality risk was higher in patients with DM compared to regional rates. This increase was seen in different types of cancer. More in detail, the risk to die from malignant neoplasms is increased in more than 30 % of diabetics, no matter which gender, and there is even a two-fold increased risk of patients with DM to die from liver cancer and pancreatic cancers. Furthermore the mortality risk from uterus cancer and breast cancer, in women with DM, was significantly higher compared to regional rates. And in men with DM, lung cancer and colorectal cancer were predominantly leading to a significantly higher risk of mortality, in comparison to regional rates (Fedeli et al., 2014). Moreover many trials focus on pancreatic cancer. In a case control study some interactions were found between different risk factors, like the association between cigarette smoking, DM and developing pancreatic cancer. However, the mechanisms behind this interaction is unknown yet (Hassan et al., 2007). In Taiwan a cohort study

was conducted in order to investigate whether subjects with DM are at higher risk to develop pancreatic cancer. There is debate as to whether DM has the potential to increases the risk of pancreatic cancer. In this investigation from Liao et al. the incidence of pancreatic cancer was significantly higher in patients which were suffering from DM less than two years, compared to non-diabetic subjects. Moreover the effects of anti-diabetic drugs were investigated too, but there was no significant association between pancreatic cancer and anti-diabetic drugs found. So the conclusion was that pancreatic cancer is associated especially with the onset of T2DM (Liao et al., 2012). And medical professionals often consider that the new onset of DM is a marker for occult cancer, or that there is even a reverse causality, so DM can be seen as a consequence of cancer (Fedeli et al., 2014). Furthermore in a study from Chari et al., the focus was also on subjects with new-onset DM. They investigated that subjects were at higher risk of being diagnosed with pancreatic cancer within the first three years after the diagnosis of DM. And approximately 1 % of diabetics (aged ≥ 50 years) will be diagnosed with pancreatic cancer within the first three years after diagnosis (Chari et al., 2005). In contrast to the Taiwan cohort study, in which no significant association between pancreatic cancer and anti-diabetic drugs have been reported (Liao et al., 2012), links were found between anti-diabetic drugs and the reduce for the risk of cancer in other studies. A systematic review by Wang et al. investigated if metformin has the potential to decrease the risk of pancreatic cancer in patients with T2DM. Ten cohort studies and three case control studies have been analyzed. This meta-analysis showed a 37 % reduction of the risk to suffer from pancreatic cancer in association with the use of metformin as an anti-diabetic drug (Wang et al., 2014). Simó et al. conducted a case-control study with 275,164 T2DM patients in Barcelona. The control group was matched by sex, age, geographical area, and diabetes duration. Treatments such as insulin, metformin, sulfonylureas (SU), thiazolidinediones and many more have been analyzed. They came to the conclusion that anti-diabetic drugs have no significant influence of cancer risk in patients with T2DM (Simó et al., 2013). In another trial, which focused on insulin treatment, no association was found between cancer incidence and the dose of human insulin or other analogues (Mannucci et al.,

2010). In Scotland three types of insulin treatments have been compared. Also here no differences between the types of insulin and no association between the treatments and an increased cancer risk have been found (Colhoun, 2009). Other outcomes in contrast indicate that insulin treatment has an association with higher cancer risk. That was reported in the EPIC study whereas participants which were treated with insulin were at a higher risk to suffer from hepatocellular carcinoma, when they were compared to participants without DM (Schlesinger et al., 2013). Further there were even some conflicting results in a small number of studies, which show that some anti-diabetic treatments were associated with a higher risk of cancer. An elevated cancer risk or death from cancer among diabetics was found in those which were treated with SU compared to those ones which were using metformin as medication (Bowker et al., 2006). As mentioned before, 387 million people are suffering from DM worldwide and this number is increasing every year (IDF, 2014a). This continuously growing number of diabetics and the higher risk for diverse types of cancer could lead to a social, a clinical and certainly to an economic burden. Therefore further investigations are needed to develop a reasonable approach for the prevention of cancer and treatment of cancer in patients with DM (Shikata et al., 2013).

2.2. DNA damage and Diabetes

Human DNA damage, is mainly caused by reactive oxygen species (ROS), which include free radicals, but also other highly reactive forms of oxygen (hydrogen peroxide, singlet oxygen, superoxide anion radical, hydroxyl radical, peroxynitrite and nitric oxide) (Azqueta et al., 2009). This damage is induced by an excess of oxidation processes in the human body and is defined as oxidative stress (Sies, 1985). In DM, metabolic factors can cause damage to the DNA. The most important mediator in damage of DNA in diabetics is probably oxidative stress. In addition accumulation of DNA damage may lead to mutations which are playing a major role in carcinogenesis. Therefore, damage of DNA is possibly an important biological link among DM and cancer (Lee and Chan, 2015).

2.2.1 Reactive oxygen species and oxidative stress

The major production of ROS takes place in the mitochondria. The biradical oxygen is needed for the respiratory chain, and therefore oxygen is transported throughout red blood cells to all individual cells. In these cells (primarily in the mitochondria) oxidation is taking place, resulting in CO_2 and H_2O formation, and energy release. Some oxygen is not utilized resulting in free radicals. Hence the majority of ROS are by-products of electron transport reactions catalyzed by the mitochondria (Latscher and Kazmaier, 2008). Endogenous sources of ROS are not only mitochondria, but also the metabolism of cytochrome P450, inflammation by neutrophils and macrophages and peroxisomes (Conner and Grisham, 1996; Gupta et al., 1997; Valko et al., 2006). Besides that there are many other mechanisms, which are involved in the formation of ROS, such as irradiation with UV light, X-rays and also gamma rays, or they can be produced during metal catalyzed reactions. Furthermore they are also present in the atmosphere as pollutants (Rahman, 2007). Another biological important process in which ROS are produced is lipid peroxidation, where peroxyl radicals are formed, which are involved in many human diseases (Gutteridge, 1995). Most free radicals consist of one or more unpaired electrons (Latscher and Kazmaier, 2008). Hence ROS are extremely unstable, reactive, and therefore harmful for biomolecules. The three main ROS are superoxide radical, hydrogen peroxide and the hydroxyl radical. Superoxide radicals ($\bullet O_2^-$) are a result out of 1- electron reduction from molecular oxygen, which leads to the formation of hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\bullet OH$) (Löffler, 2008). These species are lightly reactive, since they donate or accept electrons from other chemical compounds very easily. ROS can damage almost all biomolecules like DNA, proteins, carbohydrates, or membrane lipids, even hyaluronic acid and proteoglycane. Within the DNA, strand breaks and base modifications are initiated (Löffler, 2008). DNA oxidation, which is mainly induced by ROS, is one of the most common forms of damage to the human DNA (Azqueta et al., 2009). Oxidative stress can be seen as a pathological condition with an imbalance between formation of free radicals and their degradation (Behl et al., 2015). To reduce this damaging impacts a reduction of ROS is important for living organisms. Single strand breaks usually be repaired easily. A cellular mechanism that repairs damaged DNA is the base excision repair (BER) (Collins,

2014). Furthermore the human body is dealing with this pathological effects of ROS by utilizing non-enzymatic antioxidants, such as vitamin E, vitamin C, beta-carotene, flavonoids etc., or enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), or glutathion peroxidase (Gpx). All of them are playing an important role in reducing the damage to DNA (Löffler, 2008; Valko et al., 2006). The enzyme SOD catalyzes $\bullet O_2^-$ conversion to H_2O_2, while CAT converts H_2O_2 to H_2O and O_2 (Dalton et al., 1999). Moreover several co-factors are playing a major role for antioxidants, in order to achieve the best possible effects. These co-factors are e.g. copper, zinc, selenium, iron, coenzyme Q10 (Ubiquinone), thiamin, or cobalamin (Rahman, 2007). Oxidative stress is involved in the pathogenesis of insulin resistance, elevated blood pressure, dyslipidemia, inflammation, endothelial dysfunction and therefore to the metabolic syndrome (Tangvarasittichai, 2015). Subsequently oxidative stress triggers the development of diseases like neurodegenerative diseases, cardiovascular diseases, DM and lately to cancer (Rahman, 2007). It is associated with ageing, since free radicals are participating in biochemical, physical, and pathological alterations which are associated with the aging process. Oxidative damage to lipids, proteins and to DNA is accumulating and increasing with age (Latscher and Kazmaier, 2008; Rahman, 2007).

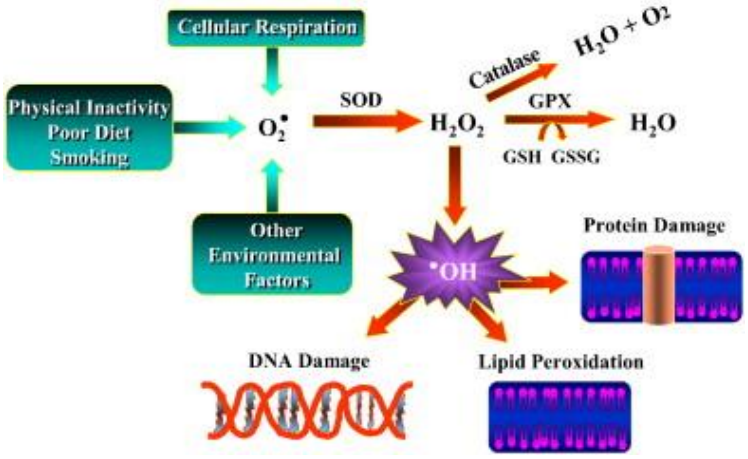

Figure 1: The generation and the detoxification of ROS (Roberts and Sindhu, 2009)

2.2.2 Oxidative stress and type 2 diabetes

DM is linked to an increase in oxidative stress. This is indicated by various factors, such as raised concentrations of lipid peroxidation products, such as MDA or conjugated diens in human plasma (Lyons, 1991). Moreover oxidative stress is a sources of non-enzymatic, enzymatic, and mitochondrial pathways. Furthermore transient changes in the activity of antioxidant enzymes (SOD, CAT and Gpx), which are involved in reduction of ROS, were found in diabetics, which were poorly controlled. Perhaps there is a direct inhibition of these enzymes by hyperglycemia (Pieper et al., 1995). ROS are involved in the development of T2DM and in complications of T2DM. It has been demonstrated that there is an association of the pathogenesis of insulin resistance and oxidative stress. This is bound on inhibitions of insulin signals and adipocytokines dysregulation (Furukawa et al., 2004). Furthermore there is an increase in ROS in the β-cells, due to the high glucose oxidation and active metabolism. In addition β-cells are at low amount of redox-regulating enzymes and free radical detoxifying enzymes, such as CAT and SOD, Gpx, and thioredoxin. The consequence out of this limited scavenging system is a rapid increase in ROS in the β-cells due to an increase in NADPH oxidase (NOX) (Newsholme et al., 2007). The metabolic abnormities in T2DM are mostly caused by the superoxide overproduction in the mitochondria, which is a consequence of intracellular hyperglycemia. This elevated production of SOD is the major mediator of tissue damage in diabetics. Hyperglycemia can induce oxidative stress by several mechanisms. There are five pathways which are involved in pathogenesis of diabetic complications. The activation of the polyol pathway, the formation of advanced glycation end-products (AGE), the elevated expression of receptors from AGEs (and also its activating ligands), the activation of protein kinase C (PKC), and the high activity of the hexoamine pathway. All of these five pathways ameliorate several diabetes-induced abnormalities. Moreover the high level of SOD inactivates two anti-atherosclerotic enzymes: prostacyclin synthase and endothelial nitric oxide synthase (eNOS) (Giacco and Brownlee, 2010). Because of these metabolic abnormalities there is evidence that oxidative stress plays a role even in the development of diabetic complications, both macrocascular and microvascular. The progression of diabetic retinopathy is one of the induced complications, since oxidative

stress can damage the retinal endothelial cells (Behl et al., 2015). To prevent more damage there is it important of optimize the individual glycaemic control. The reduction of damage to DNA by the use of alternative drugs or antioxidants may be one target (Lee and Chan, 2015). So dietary antioxidants, as supplements, or foods which are rich in antioxidants, have been studied to investigate whether oxidative damage is influenced (Møller and Loft, 2006). Different outcomes were reported, so there are some trials in which no association was found in antioxidants and DNA damage (Sampson et al., 2001), but there are other outcomes with a positive effect of antioxidants on DNA damage (Müllner et al., 2013; Şardaş et al., 2001).

2.2.3 Oxidative stress and cancer

ROS induced DNA damage leads to mutations, and thereafter, to the etiology of different degenerative diseases like cancer. In the nuclear DNA a great number of oxidized bases have been identified. 8-oxo-7,8-dihydroguanine (8-oxoGua) is definitely one of the most common and readily formed DNA lesion. It has been suggested that this type of lesion plays a key role in all processes of carcinogenesis, the initiation, the promotion and the progression of a tumor. Hence 8-oxoGua may lead in human cells to mutations (Cooke et al., 2003). 8-oxoGua is formed relatively easy and could be a good biomarker of human carcinogenesis (Valko et al., 2006). In prostate cancer patients data suggests that the damage of DNA is largely related to oxidative stress, indicated by the oxidation of the base guanine, 8-oxo-dG (8-oxo-2- deoxyguanosine). This base oxidation has been occurred in the human prostate of the majority of men which underwent a prostate biopsy (Wu et al., 2009). The key repair enzyme for oxidized guanins in the DNA of eukaryotes is 8-oxoGua-DNA glycosylase (OGG1), which is involved in BER. Other relevant repair enzymes are e.g. MutY homologue (MUTYH), MutT homologue (MTH1), endonuclease III homologue 1 (NTH-1), and human Nei-like (NEIL) DNA glycosylases (Bai et al., 2007; Hazra et al., 2007; Nakabeppu, 2001). A high repair capacity may lead to a lower level of DNA damage and therefore the cancer risk can be reduced, but otherwise a higher repair rate may reflect also a higher exposure to agents that damage DNA (Collins, 2014). Another important part in the elimination of DNA damage is Mn-SOD (manganese superoxide dismutase), which is a very

powerful antioxidant enzyme which has an anti-tumor function. There is indication that an imbalance between the formation of superoxide radicals and the degradation of hydrogen peroxide occur in cells, which show an overexpression of Mn-SOD, which may activate a metastatic potential on cancer cells (Valko et al., 2006). Besides that facts, ROS can mediate many pathways for cell-signaling, which are then involved in pathways that regulate cell growth and are therefore involved in carcinogenesis. The activation of different transcription factors, as MAP-kinase/AP-1 or NF-B pathways, has then a direct impact on cell proliferation and apoptosis. Hence damage to DNA, modified gene expression and mutations are altogether key actors in carcinogenesis (Valko et al., 2006). To prevent the cancer risk there is indication that a combination of antioxidants may be a useful approach (Eli and Fasciano, 2006). But this should be considered with caution, because several antioxidants, such ascorbic acid or beta-carotene may also act as pro-oxidant in high concentrations (ATBC, 1994), which might stimulate the tumor growth throughout increased survival of the tumor cells (Valko et al., 2006).

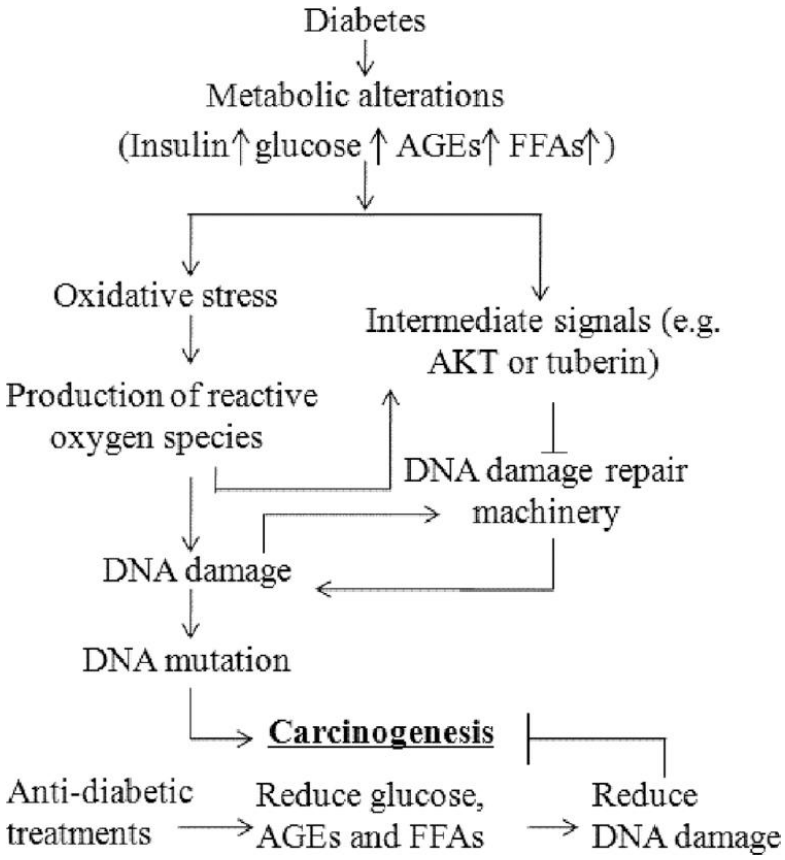

Figure 2: Pathways connecting DM and DNA damage which are involved in carcinogenesis (Lee and Chan, 2015)

2.3 Comet assay

2.3.1 Comet assay

DNA damage can be verified by using the comet assay or also called single cell gel electrophoreses. Thereby single cells are embedded in low-melting-point agarose on a microscope slide (Singh et al., 1988). Cells are treated with a lysis solution followed by electrophoresis and fluorescence microscopy for evaluation (Olive et al., 1991). To assess DNA damage this method has become one of the most commonly used methods (Azqueta et al., 2009). The comet assay has its roots in the 1970s by Peter Cook and his colleagues. At that time a method was developed in order to investigate nuclear structures which were exposed to agents that can damage DNA (Cook et al., 1976). Based on that Ostling and Johanson developed this method further. They designed a microgel electrophoresis assay on cells, which were irradiated with γ-rays,

embedded in agarose and lysed with a neutral detergent solution. An electric field was applied and because of the negative phosphate end DNA strand breaks migrated towards the anode. The typical visual appearance of a comet was analyzed under a fluorescent microscope (Ostling and Johanson, 1984). Additional changes were made by Singh *et al*. (Singh et al., 1988) followed by Olive *et al*. who developed a version, under neutral or alkaline conditions (pH = 12.3) (Olive et al., 1991). Lately changes were performed by Azqueta et al. (Azqueta et al., 2009). Today the comet assay is a widely adopted method (Neri et al., 2015). Due to its rapidity, sensitivity, simplicity and of course because it is a cost-effective way to detect DNA damage (Collins et al., 1997). Therefore the comet assay has become the method of choice in order to detect DNA damage (Collins, 2014). It is applied in human biomonitoring studies (Collins et al., 2014) or genotoxicity testing (Hartmann et al., 2003) and even used to study different effects of/or factors that can contribute to diseases (Dusinska and Collins, 2008). Different forms of DNA damage can be analyzed. Single-strand DNA breaks, double-strand DNA breaks and alkali labile sides can be measured with the alkaline comet assay (Collins et al., 1997). The basic comet assay was modified to detect also oxidized bases, by the introduction of an incubation time of nucleotides after lysis by means of bacterial repair enzymes. In order to measure these oxidized bases either formamidopyrimidine glycosylase (FPG) or endonuclease III (Endo III) is used. FPG recognizes oxidized purines while Endo III recognizes oxidized pyimidines (Collins et al., 1993; Dusinska and Collins, 2008). Besides treating cells with enzymes they can also be challenge with H_2O_2. In this case strand breaks are induced, which depends on the individual level of antioxidant defenses in the cells (Collins, 2014). In literature there is a great number of dietary intervention trials where the comet assay was conducted. Many trials showed a decrease in DNA damage, e.g. by the regular ingestion of multivitamin tablets (Duthie et al., 1996), antioxidant supplementation (Şardaş et al., 2001) or kiwifruit (Collins et al., 2003). There is also a great number of human biomonitoring studies in which the comet assay has been applied. Seasonal variations in comet scores have been detected (Smolková et al., 2004). Environmental influences on DNA damage like sunlight exposure were evaluated too by the use of the comet

assay (Moller et al., 2002). Moreover the impact of lifestyle factors were measured (Giovannelli et al., 2002; Hardoon et al., 2010). In many investigations humans PBMC'S (peripheral blood mononuclear cells) were used. But other cells or tissue can be used as well, as buccal epithelial cells, sperm, nasal epithelial cells, whole blood or adipose tissue (Chuang and Hu, 2004; Fortoul et al., 2003; Jones et al., 2014; Schmid et al., 2007; Szeto et al., 2005; Trzeciak et al., 2012). These are just a few of the options, but most commonly PBMC'S and whole blood are used (Al-Salmani et al., 2011). PBMC'S are not typical somatic cells, but because of the fact that they circulate all over the body, their nuclear, cellular and metabolic state reflects the whole body exposure (Dusinska and Collins, 2008).

2.3.2 Comet assay and oxidative stress

Oxidative stress as cause or effect is one main factor in the development of many diseases. Therefore the products of DNA oxidation are a good biomarkers to investigate the impacts of oxidative stress on human cells. Furthermore, there is the benefit that DNA oxidation products are relatively simple to measure. And oxidized DNA bases are an indicator of the redox state of the entire organism, so they can represent a valuable marker in trials of many chronic diseases (Collins and Dusinska, 2002; Dušinská et al., 1999). The comet assay has become the method of choice for the measurement of DNA damage, inclusively oxidative damage caused by ROS. Like mentioned above oxidative attacks can be indicated more specifically by oxidized pyrimidines or purines, recognized by FPG or Endo III. More precisely, FPG recognises 8-oxoGua, and even ring-opened purine derivatives, or formamidopyrimidines, such as 2,6-diamino-4- hydroxy-5-formamidopyrimidine (FaPyGua), and 4,6-diamino-5-formamidopyrimidine (FaPyAde). Endo III acts on oxidized pyrimidines, like thymine glycol and also uracil glycol. This happens by a glycosylase activity that is combined by these enzymes (endo III and FPG), which remove the damaged base and create an AP-site (apurinic/apyrimidinic), and analyses specific for AP, which transforms these AP site to a DNA break. These enzymes are available commercially, or they can be isolated from over-producing strains of bacteria. Oxidized bases can be indicated by an increase in tail DNA (%) after the incubation process with the enzyme, compared to buffer

alone (Collins, 2014). Furthermore it is also possible to measure repair pathways by comet assay. 8-oxoGua can be induced through treating cells by the photo sensitizer Ro 19-8022 and light, and the pathway for repairing is the BER, with OGG1. Repair enzyme carries out removal of this oxidized base and also cleavage at the AP-site (Collins, 2014).

2.3.3 Comet assay and Diabetes Type 2

A multitude of studies have been conducted to investigate the impact of DNA damage on DM by the use of the comet assay. Many trials came to the conclusion that patients with DM show higher DNA damage compared to healthy subjects (Collins et al., 1998; Lodovici et al., 2008; Tatsch et al., 2012; D. J. Xavier et al., 2014). In contrast to that Ibarra-Costilla et al. did not find significant differences in DNA damage levels, which were evaluated by comet assay, when healthy controls were compared to patients with T2DM (Ibarra-Costilla et al., 2010). Besides comparing diabetics to healthy controls, the evaluation of individual differences in DNA damage in-between subjects with DM is another point of interest. Xavier et al. reported that hyperglycemic T2DM patients showed higher levels of baseline DNA damage and oxidative DNA damage in PBMC'S than non hyperglycemic T2DM patients or healthy controls. But interestingly there was no significant differences between the non hyperglycemic T2DM and the healthy controls, what means that these two groups show similar levels of both baseline DNA damage and oxidative DNA damage (D. J. Xavier et al., 2014). Moreover, higher fasting glucose in T2DM patients is associated with higher DNA tail damage (Tatsch et al., 2012). Also of interest is the association between FPG sensitive sides and blood glucose level of subjects with T2DM, because positive correlations were found in FPG sensitive sides and serum glucose and blood glucose (Collins et al., 1998; Dincer et al., 2002). Furthermore differences have been investigated in DNA migration when patients with insulin dependent diabetes mellitus (NIDDM) were compared to patients with IDDM (insulin depended diabetes mellitus). Results showed that patients with NIDDM had significantly higher DNA damage compared to IDDM. The authors concluded that this results could indicate that IDDM patients are handling more oxidative DNA damage on a regular basis (Şardaş et al., 2001). Moreover lifestyle and

diet are heaving a huge impact on the development of T2DM and therefore also on DNA damage (Hardoon et al., 2010). Hence lots of trials have been conducted in order to investigate supplementation of macronutrients or micronutrients. For example Xavier et al. investigated the impacts of a seven-day hospitalization period on DNA damage patients with T2DM in order to achieve adequate blood glucose levels by a dietary intervention in combination with medication treatment. This seven-day period at the hospital with the aim of improving glycaemic control had significant ($p < 0.05$) effects on DNA damage levels in peripheral blood cells from patients with T2DM (Danilo J. Xavier et al., 2014). An antioxidant supplementation with vitamin E was conducted from Şardaş et al. Patients with IDDM (n=63) and patients with NIDDM (n=48) and also age-matched healthy controlled (n=30) where treated twelve weeks with either vitamin E (900 mg/day) or placebo. Comet assay was conducted on isolated lymphocytes. Results showed a significant ($p < 0.05$) decrease in the DNA migration in all treated patients in comparison to their mean DNA migration before treatment (Şardaş et al., 2001). Furthermore, Müllner et al. conducted a randomized intervention trial in which patients with 76 subjects with DM (type 1 and type 2) and a control group of 21 subjects received 25 ml PUFA-rich plant oil and additionally 300 g of vegetables per day for the duration of eight weeks. DNA Strand breaks, sensitivity to H_2O_2, and FPG-sensitive sites were measured in PBMC'S by the comet assay. After eight weeks this trial resulted in a significant reduction of DNA strand breaks by 17.1 % ($p < 0.001$) in diabetic patients (Müllner et al., 2013). In some studies the differences between patients with T2DM were evaluated. In Mexico a case control study on subjects with T2DM was conducted. All of them were under oral treatment (SU and/or metformin), and they showed poor glycaemic control. DNA damage in leukocytes was analyzed by the comet assay. Three age groups were formed and all groups show similar DNA damage, there was just a slightly higher damage in the two younger groups compared to the oldest (Ibarra-Costilla et al., 2010). Another parameter to distinguish is the level of HbA1c. DNA damage is considered to be higher in patients with poor glycaemic control compared to a good glycaemic control. Xavier et.al. reported a significant difference in the DNA damage in T2DM patients with higher

HbA1c compared to patients with lower HbA1c (Xavier et al., 2015). Moreover the metabolic syndrome (MetS) is another interesting issue, because T2DM is one of its risk factors. And risk factors for MetS occur more often together than separately (Alberti et al., 2009). So the evaluation of DNA damage in connection with MetS is another emerging field in which comet assay is conducted. Significant differences in the comet tail length were found when patients with MetS where compared to healthy subjects. That means that patients with MetS show higher DNA damage. In addition a correlation between HbA1c and tail length was found (Karaman et al., 2015). But in contrast Mili´c et al. conducted a study on patients in the early stage of MetS. In this case no significant changes were found in DNA damage (tail intensity, tail moment, tail length) when MetS patients where compared to healthy subjects (Milic et al., 2015).

2.3.4 Comet assay and cancer

The comet assay may also be an appropriate technique to assess the early cancer risk, throughout measuring DNA damage in human lymphocytes (Collins et al., 2014). DNA damage and also DNA repair capacity are molecular events, which are driving cancer initiation and cancer progression. Because of this fact the comet assay has been used in various studies to investigate DNA damage. Studies have been conducted in a wide range of cancer cells, like cells which were extracted from human tumor biopsies or peripheral blood lymphocytes (McKenna et al., 2008). Various studies have already shown a connection between higher DNA damage and different types of cancer, especially when cancer patients were compared to healthy subjects (Collins et al., 2014). A study on women with breast cancer was conducted from Santos et al. They recruited 45 women with breast cancer, which were under no medication that causes additional DNA damage and which were free from any other pathology. These patients were compared to 85 age-matched female controls. Breast cancer patients showed significantly higher tail intensity in lymphocytes compared to healthy controls. So the authors concluded that untreated breast cancer patients have higher levels of DNA damage compared to healthy subjects (Santos et al., 2010). Similar results were found by Synowiec et al. who collected blood from breast cancer patients before therapy and from age-matched healthy controls. The mean level of basal damage (% DNA in tail)

was higher in patients with breast cancer compared to healthy controls, however this was not significant. Oxidative DNA damage (% DNA in tail), which was assessed throughout the enzymes Endo III and FPG, was significantly higher in breast cancer patients compared to healthy controls (Synowiec et al., 2008). Further there was a link between carcinoma of the cervix and DNA damage. Patients with carcinoma of the cervix showed significantly higher damage to DNA compared to healthy controls (Gabelova et al., 2008). Another working group found a significant increase in DNA damage in esophageal tissue cells and blood cells in patients with esophagus carcinoma, in South Asia (Vasavi et al., 2010). A connection was also seen in subjects with ovarian cancer. In this trial 30 women which were suffering from ovarian cancer were compared to twenty healthy females. Blood was taken before of any therapy, and PBMC'S were isolated. Results showed that there was a significant difference in damaged cells between cancer patients and healthy controls. Therefore the authors concluded that the comet assay is a successful method to monitor DNA damage in women with ovarian cancer (Baltaci et al., 2002). Also in patients with Hodkin's disease a higher DNA damage was observed, when these patients were compared to healthy subjects (Lorenzo et al., 2009; Pavlov et al., 2010). However there were also studies showing no differences in DNA damage between cancer patients and healthy subjects. Sigurdson et al. measured the amount of DNA single-strand breaks in individual cells by using the comet assay and reported that there were no significant associations with lung cancer found when comparing lung cancer patients with controls (Sigurdson et al., 2011). Another way is to challenge cells with radiation, or to investigate the effects of chemotherapy and radiotherapy (McKenna et al., 2008). Blasiak et al. conducted a trial in breast cancer patients, where PBMC'S % of DNA in tail was measured. Their results showed that women with breast cancer had significant higher DNA damage compared to healthy subjects. And additionally they demonstrated that DNA damage was significantly higher after chemotherapy, in cancer patients. The DNA damage after chemotherapy was even too high to measure it properly in some cases. They concluded that the comet assay may be adopted to evaluate the potential of a particular mode of chemotherapy to a cancer patient (Blasiak et al., 2004).

3 Materials and Methods

3.1 Study Design

This study was a cross-sectional study considering females with T2DM. The study was performed as a cooperation between the Department of Nutritional Sciences of the University of Vienna, and the Diabetes Outpatient Clinic at the Vienna Regional Health Insurance Centre-South. The duration of the study was four months. From Mai to September 2014 samples were taken on Mondays and Tuesday, within the routine check of the patients at the DM ambulance. Prior to the sampling, all subjects were recruited during their routine check at the Diabetes Outpatient Clinic. Only women were included in this study in order to exclude gender-specific differences and to reach more statistical power. They were all treated with oral anti-diabetics and/or insulin.

3.2 Study Population

Hundred forty five female subjects with diagnosed T2DM were included in the study. Females with T2DM who met the inclusion criteria (table 3), were enrolled. A set of exclusion criteria are shown in table 4. Eight subjects were excluded of the study bound on this criteria. Out of these subjects five were still smokers, two had a HbA1c which was too low, and one showed poor kidney values. Thus 146 subjects were included into the study.

Table 3: Inclusion criteria

- *Females with Type 2 Diabetes Mellitus (T2DM)*
- *Age: over 30 years*
- *Medication: oral anti-diabetics and/or insulin therapy*
- *Last four weeks prior starting the study: Constant nutritional behavior, constant physical activity, constant weight*
- *Non-smoking for at least 1 year*

Table 4: Exclusion criteria

- *Patients with type 1 diabetes mellitus (T1DM)*
- *Male*
- *Age: below 30 years*
- *Pregnant or lactating women*
- *Participation in another clinical trial*
- *Within the last 4 weeks prior starting the study: Change of medication which influence metabolic parameters*
- *Significant cardiovascular damage with NYHA > III*
- *Liver disease with three-times higher transaminase values*
- *Chronic kidney disease with serum creatinine > 2 mg/dl*
- *Dialysis*
- *HIV positive*
- *History of chronic alcohol abuse in the last two years*
- *History of cancer, stroke, organ transplantation*

The study was approved by the Ethics Committee of the Medical University of Vienna (EK Nr: 1987/2013). The study is listed at ClinicalTrials.org (NCT02231736).

3.2.1 Anthropometric parameters

The anthropometric parameter, which were measured on the study day, included weight (kg), height (m), waist and hip circumference (cm) abdominal girth (cm) and blood pressure (mmHg). Out of these measurements the BMI was calculated.

3.2.3 Chemical parameters

All biochemical parameters were measured at the Diabetes Outpatient Clinic (Table 5).

Table 5: Biochemical parameters measured within the study

DIABETES PARAMETER	*blood glucose (mg/dl)*
	HbA1c (% and mmol)
	insulin (uU/ml)
	c-peptide (ng/ml)
LIPID METABOLISM	*cholesterol (mg/dl)*
	HDL-cholesterol (mg/dl)
	LDL-cholesterol (mg/dl)
	cholesterol-HDL-ratio
	LDL-HDL-ratio
	triglycerides
PROTEINS	*c-reactive protein (mg/l)*
	total protein (g/dl)
URIN ANALYSIS	*blood urea nitrogen (mg/dl)*
	estimated glomerular filtration rate (ml/min)
	uric acid (mg/dl)
	creatinine (mg/dl)
HORMONS	*cortisol (in the morning) (ug/dl)*
	thyreoida stimulating hormone (uU/ml)
ENZYMES	*creatine phosphokinase (U/l)*
	glutamate-oxalacetate-transaminase/aspartate-aminotransferase (GOT/ASAT)
	glutamate-pyruvate transaminase/alanin

	aminotransferase (GPT/ALAT)
	gamma-glutamyl-transferase (U/l)
VITAMINES	*iron (ug/dl)*
	ferritin (ng/dl)
	B-vitamins: cobalamin (pg/dl) and folic acid (ng/dl)

3.3 Blood sampling and isolation of PBMC'S

Blood sampling took place on the Diabetes Outpatient Clinic. In addition to blood, urine, saliva and buccal cells were collected. In total 45 ml EDTA blood was sampled after an overnight fasting. The blood samples were transported ASAP in a cooling box to the Department of Nutritional Science in Vienna. The preparation of the blood took place under lamina flow. At first, 2 ml of whole blood were pipetted in tubes from every subject. Out of that, two times 100 µl of whole blood from every subject were treated with nitrogen and then cooled down slowly and stored at -80°C, for the comet assay with whole blood. Subsequently the remaining whole blood was transferred in the leucosep tubes, again two tubes per subjects. These tubes were centrifuged for 15 minutes at 1000 x g with turned off brakes at room temperature. After centrifugation all subsequent processing steps took place on ice. The obtained lymphocyte suspension was transferred in a new 50 ml tube and was filled up to 15 ml with cold PBS (phosphate-buffered saline). Tubes were mixed five times over head. Thereafter samples were centrifuged again for 15 minutes at 259 x g with switched-on brake at 4°C. After this centrifugation step the supernatant was removed and the cell pellet was resuspended carefully in 1 ml of PBS and thereafter refilled with another 9 ml of PBS. Tubes were mixed five times over head again and samples were centrifuged ones more under the same conditions (259 rcf/g = 1300 rpm, 10 min, 4°C, with brake). Afterwards the supernatant was removed a second time and the cell pellet was resuspended carefully again with 1 ml of PBS. And the two tubes per subjects were combined now into one. During the following step the cell number was determined via an automated cell counter (countess-). Therefore 60 µl PBS + 20 µl cell suspension were mixed. Out

of that cell suspension 10 µl were taken and mixes with 10 µl trypanblue. Thereof 10 µl were placed in a plastic chamber of a special slide for the countess. For determining the cell number the countess was focused and the number of living and dead cells were counted and noted. For the comet assay two times 0.5x10^6 cells (PBMC) were calculated per every subjects. Samples were mixed with 0.5 ml freezing medium (FBS+10%DMSO) and thereafter slowly cooled down and stored at -80°C.

3.4 Principles of the comet assay

The comet assay is a widespread, simple and sensitive method for detecting DNA damage. In this study comet assay was performed in PBMC'S and in whole blood. The assay gives information about single-strand breaks and double-strand breaks. Throughout the enzyme FPG one get information about oxidized purines too. A single-cell suspension was embedded in LMA on a microscope slide, which was coated with NMA. Cells were lysed to remove the cell contents except for nuclear material. Thus proteins, membranes, nucleoplasmic constituents and cytoplasm were removed and the DNA remained highly supercoiled without histones (Singh et al., 1988). Then cells were placed in alkali conditions where the DNA begins to unwind. Thereafter an electric current was applied and the damaged DNA extends towards the anode. So an appearance of a "comet tail" was given. In contrast undamaged DNA remained in the head (Collins et al., 1995). Gels were stained with GelRed and tail intensity was evaluated under a fluorescence microscope.

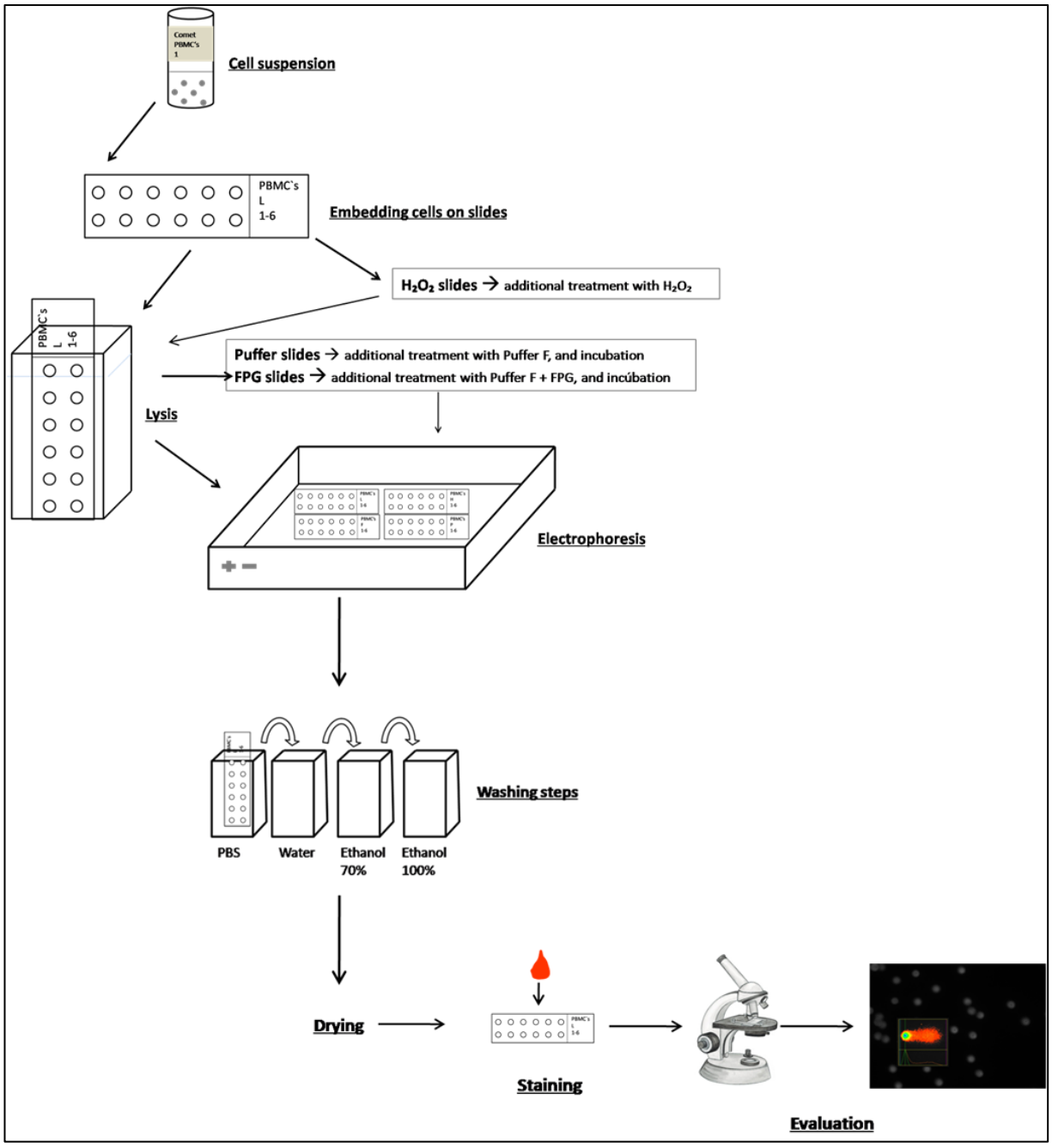

Figure 3: Scheme of the comet assay procedure

3.4.1 Comet assay procedure

The protocol for PBMC'S, is based on Azqueta et al. (Azqueta et al., 2009), with slight modifications. The protocol which was used for the comet assay with whole blood was based on Al-Salmani et al. (Al-Salmani et al., 2011), also with slight modifications. We performed a 12 gel method and some additional washing steps were introduced in

order to optimize the method. Chemicals, equipment and the detailed protocol can be found in the Appendix.

3.4.1.1 Slide preparation

NMP Agarose was mixed with double distilled water and heated slowly in the microwave until agarose was dissolved and the fluid was clear. Water bath was heated up to 55°C. The vessel with the fluid agarose was placed in the water bath. Slides were slowly dipped into the agarose and the reverse side of the slides were wiped off on a paper towel. Then the slides were dried up overnight at room temperature. The detailed protocol is listed in the appendix. All coated slides were stored in a box at room temperature. The remaining agarose was filled in small bottles and kept in the fridge at 4°C.

3.4.1.2 Washing the lymphocytes

This procedure needed to be done to remove freezing medium. Samples were taken out of the freezer at -80°C. Then they were thawed quickly in the hand or in a water bath at 37°C. Immediately after thawing, samples were centrifuged, and placed on ice and the supernatants were removed. The remaining cell pellets were resuspended carefully with 1 ml of PBS. All these steps needed to be done fast, because the freezing medium contains DMSO, which reacts an a cytotoxin at room temperature. Samples were centrifuged again. The second time, the supernatants were removed and cell pellets were resuspended carefully with 300 µl of PBS. Cell number was determined by the countess (Invitrogen), an automated cell counter. Living and death cells were counted as well as the viability was determined. For each sample a concentration of 6 x 10^5 living cells was calculated. The detailed protocol can be found in the appendix.

3.4.1.3 Spreading cells on slides

Procedure with PBMC'S:

The slides which were coated with NMA, were labeled (figure 4). The 12 gel method was conducted. So one slide contained six subjects, two gels per subject.

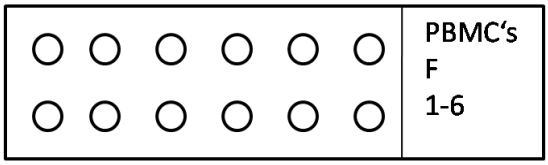

Figure 4: Labeling of slides. This figure shows a slide with PBMC'S from subjects 1 to 6. "F" stands for the treatment with FPG.

Four slides (lysis, H_2O_2, FPG, buffer) were prepared each with six subjects. In addition four identical backup slides were prepared too. LMA was heated and placed in the water bath at 37°C. Metal plates were placed on ice and the slides were placed on this plates. Then cell suspension was mixed in a cup with NMA and immediately spreaded on the slides. Eight times 5 µl per subject were placed on the four slides. The detailed protocol can be found in the appendix. Throughout the small amount, gels were stocked immediately after spreading on the cold coated slides.

<u>Procedure with whole blood:</u>

The slides, which were coated with NMA, were labeled (figure 5). Even in this case the 12 gel method was conducted.

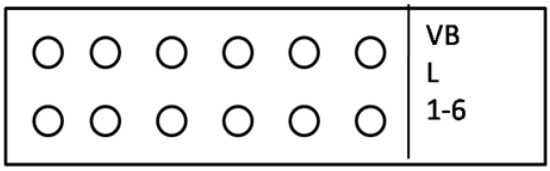

Figure 5: Labeling of slides. This figure shows a slide with whole blood (VB) from subjects 1 to 6. "L" stands for the treatment with lysis.

Therefore there were three slides (lysis, FPG, buffer) each with six subjects. In addition three identical backup slides were prepared. LMA was heated and placed in the water bath at 37°C. Metal plates were placed on ice and the slides were placed on this plates. Whole blood and LMA were mixed in a cup and in each case 5 µl of the mixture were placed on the coated slides. That means six times 5 µl per subject. The detailed protocol can be found in the appendix. Throughout the small amount, gels were stocked immediately after spreading on the cold coated slides.

3.4.1.4 Treatment with H_2O_2 and lysis

The slides, which were labeled with H_2O_2 were placed in a H_2O_2-solution for five minutes. Meanwhile all other slides were placed in the lysis solution. After five minutes H_2O_2 slides were taken out of the H_2O_2 solution and were dipped three times shortly in PBS, to remove H_2O_2. Then these slides were placed side by side to the other slides in the lysis solution. All slides were incubated one hour in the lysis solution at 4°C. The detailed protocol can be found in the appendix.

3.4.1.5 Enzyme treatment

The slides, which were labeled with "buffer" and "FPG" were taken out of the lysis solution and underwent three washing steps with buffer F. The remaining buffer was dabbed off on paper towel and the slides were placed again on the metal plates and were covered with a special silicon gasket (with twelve holes). The holes of the silicon gasket had to be placed exactly on the twelve gels of the slides. This working step needed to be done very carefully not to lose gels. On the top a hard plastic cover, with twelve holes, was placed and all parts were fixed with iron bolts. Thereafter it was placed on ice immediately. Subsequently 30 µl of FPG were pipetted on every gel of the slides, which were labeled with "FPG". And 30 µl of buffer F were pipetted on every gel of the slides, which were labeled with "buffer F". A silicon cover came on the top, and all treated slides were incubated for 30 minutes at 37°C. Meanwhile the slides which were labeled with "lysis" and "H_2O_2" remained in the lysis solution until the incubation period was over. The detailed protocol can be found in the appendix.

3.4.1.6 Alkaline treatment and electrophoresis

At the cooling laboratory "FPG" and "buffer F" slides were removed of the moist box and "lysis" and "H_2O_2" slides from lysis solution and were wiped off on a paper towel. All slides were placed side by side in the electrophoresis tank. Gaps were filled with blank slides. The electrophoresis buffer, which was already cooled down to 4°C, was added until the slides were completely covered. Electrophoresis was covered and left for a 20 minutes unwinding phase. Thereupon a current was supplied and the electrophoresis was started for 30 minutes at 25 V. The detailed protocol can be found in the appendix**.**

3.4.1.7 Washing steps

All slides were removed of the electrophoresis and four consecutive washing steps were conducted. First all slides were placed for five minutes in PBS, then for five minutes in double distilled water, then for fifteen minutes in 70% ethanol and in the final step for fifteen minutes in pure ethanol. After all, slides were dried over night in a dark place at room temperature. The detailed protocol can be found in the appendix.

3.4.1.8 Staining and quantification

Gelred was used for staining, because it is non-toxic, non-mutagenic and specifically designed to stain DNA. The stained slides were counted immediately after staining and were kept in the dark during the counting. First six gels were stained, covered and counted and thereafter the remaining six gels were stained, covered and counted. To evaluate DNA damage, Comet 5.5 image analysis software was used. This software was linked to a fluorescent microscope. Tail intensity was recorded (% tail DNA). One hundred cells per sample were randomly scored, or more precisely 50 cells per gel. The mean value was calculated for every 50 cells and than the average of these two mean values was calculated. The detailed protocol can be found in the appendix.

3.5 Statistical analysis

SPSS for windows was used for the statistical evaluation of the data. An outlier-test was carried out. One Sample Kolmogorov-Smirnov-Test was performed for checking normal distribution. Based on that Spearman or Pearson correlation was calculated. The data for HbA1c, duration of diabetes, insulin and HDL was analyzed by Spearman since these data was not normally distributed. The remaining data was analyzed by Person Correlation. Independent T-Test for normally distributed data and Mann-Whitney U Test or Kruskal-Wallis Test for not normally distributed data was carried out to analyze differences of groups. Moreover an Oneway ANOVA was performed to determine changes within the groups. Findings were considered significant at $p \leq 0.05$.

4 Results and discussion

4.1. Clinical characteristics of the study group

In this cross-sectional study data was obtained from 146 women with T2DM. The subjects were either under oral anti-diabetics and/or insulin therapy. All of them were non-smokers for at least a year. At the day of sample collection clinical parameters were obtained (table 6).

Table 6: Baseline characteristics of the subjects (n=146)

Mean age (years) ± SD	67 ± 9,9
Mean body weight (kg) ± SD	87.9 ± 20
Mean body mass index (kg/m^2) ± SD	35 ± 7.6
Mean duration of T2DM (years) ± SD	14.4 ± 8
Mean venous glucose (mg/dl) ± SD	162 ± 38.1
Mean HbA1c (%)± SD	7.8 ± 1.3
HbA1c ≤ 7,5 (n)	74
Mean HbA1c ≤ 7,5 (%)± SD	6.9 ± 0.5
HbA1c > 7,5 (n)	72
Mean HbA1c > 7,5 (%)± SD	8.9 ± 1.3
Insulin treatment (n)	60
SU treatment (n)	38
Insulin + SU treatment (n)	94
Metformin (n)	113
Glinide	6
Acarbose	2
Alogliptin	77
GLP 1-4 glucagon like protein	14
Met+alogliptin	64
Pioglitazon	17
Sgltsodium-glucose cotransporter	16

Metabolic syndrome (n)	140 (*31)

**All 5 characteristics of the metabolic syndrome (MetS) (according to IDF)*

The duration of diabetes was defined as the period from the diagnosis (diabetes onset) until the enrollment in this study

As expected, the mean BMI of the female subjects in this study was high (35 ± 7.6 kg/m²). According to WHO, a BMI greater or equal than 30 is classified as obesity. Due to that 112 subjects were obese with a mean BMI of 37.6 ± 6.7 kg/m². So the link between obesity and DM was proven. A raised BMI is one of the major risk factors for the development of non communicable diseases like T2DM (WHO, 2015). There is a complex inter-relationship between obesity, diabetes and the risk of cancer. This is related to excess insulin and constant energy supply (Guevara-Aguirre and Rosenbloom, 2015). Furthermore 140 out of 146 subjects were suffering from MetS. So we agree with Alberti et al. who described that most patients with T2DM are suffering from the MetS (Alberti et al., 2009). According to IDF, a person is defined as having MetS if he/she has central obesity plus any two of the following criteria: raised triglycerides, reduced HDL cholesterol, raised blood pressure or raised fasting plasma glucose (IDF, 2006). According to that 31 subjects in our study met all of these 5 criteria. The strong connection between MetS and DM is shown in this investigations. The mean HbA1c was 7.8 ± 1.3 %. 74 subjects had a HbA1c ≤ 7.5 % and 72 subjects HbA1c > 7.5 %. People who are poorly controlled, are reported to have a HbA1c of 8.0 % or above. People who are well controlled, are reported to have a HbA1c of less than 7.0 % (Silink, 2007). In our study group 51 females had a HbA1c ≥ 8 %, so these ones could be defined as poorly or not optimally controlled, and 33 females had a HbA1c lower than 7 % which shows good control.

4.2. DNA damage in PBMC'S and whole blood

To measure the DNA damage the comet assay was used. Strand breaks were measured by the treatment with lysis and the resistance to H_2O_2 was measured by an additional treatment of cells with H_2O_2. The enzyme modified comet assay for detecting oxidized purines was conducted too, by the use of FPG. The relative tail intensity (in %) reflects the level of the DNA damage. The percentage of DNA in tail for the treatments lysis and FPG were evaluated in PBMC'S and in whole blood and the resistance to H_2O_2 induced DNA damage (% DNA in tail) was evaluated in PBMC'S only (table 7).

Table 7: Mean DNA damage (% DNA in tail) in PBMC'S and whole blood

	Mean % DNA in tail %±SD%
Lysis:	
PBMC'S	6.23 ± 3.09
Whole blood	8.47 ± 5.30
FPG:	
PBMC'S	4.66 ± 2.70
Whole blood	5.55 ± 4.08
H_2O_2:	
PBMC'S	14.35 ± 4.66

Xavier et al. reported a mean level tail intensity from 6.69 ± 3.68 % in ten Brazilian subjects (seven females, three males) with T2DM (age 46.4 ± 12.3 years). This was evaluated by comet assay in whole blood (D. J. Xavier et al., 2014). In comparison our mean levels were 8.47 ± 5.30 %, but our subjects had a higher age 67 ± 9,9 years and there were some varieties in the methods since we used the 12-gel method and not the two gel-method. Furthermore Xavier et al. applied a longer duration of lysis. But to compare these levels of DNA damage to healthy subjects Xavier at al. reported from a

mean tail intensity from 2.57 ± 1.37 % in sixteen healthy subjects (D. J. Xavier et al., 2014). So this levels can indicate that the DNA damage in our T2DM subjects is higher compared to levels from healthy subjects from other studies. So we can agree with previous findings that DNA damage in T2DM patients is higher compared to healthy subjects (Dincer et al., 2002; Tatsch et al., 2012; D. J. Xavier et al., 2014). Our results show a higher mean DNA damage in whole blood compared to PBMC'S. In contrast to our findings Pitozzi et al. reported that DNA stand breaks in isolated mononuclear leukocytes, from 14 T2DM patients (nine males, five females) with a mean diabetes duration of 61.6 ± 4.5 years and a fourteen controls, were higher compared to the damage level in fresh whole blood from the same subjects (Pitozzi et al., 2003). Moreover, in whole blood nucleated cells consist mainly of neutrophils (about 60–75 %) and of fewer lymphocytes (about 20–30 %). Since neutrophils are just short-lived cells, analysis of damage to DNA in whole blood may be difficult to compare with lymphocytes, which have a slower turnover. Using whole blood for the comet assay is definitely simpler, but it leads to a cell population that is less homogeneous and problems can occur due to red cells appearance (Collins et al., 2014). Furthermore the differences in DNA damage in whole blood and PBMC'S can be caused by the fact that the visual scoring was conducted by two different persons, since the visual scoring is also user dependent (Forchhammer et al., 2008). So there are some influences, which could lead to differences in these outcomes. In our subjects the mean DNA damage in PBMC'S from lysis treatment was 6.23 ± 3.09 % and from H_2O_2 treatment it was 14.35 ± 4.66 % DNA in tail. We compared our results to those of Müller et al., who evaluated the % DNA tail of NIDDM patients by using also the comet assay. At the baseline they reported from a mean DNA damage from 5.25 ± 1,47 % in PBMC'S, which were treated with lysis (Müllner et al., 2013). These results were similar to ours (6.23 ± 3.09 %). The H_2O_2 induced DNA damage was about 23.50 ± 11.22 % DNA in tail (Müllner et al., 2013). In this case our results showed lower levels of DNA damage, induced by H_2O_2 (14.35 ± 4.66 %). Cells were treated with H_2O_2 in order to assess their resistance to oxidant challenge, since H_2O_2 induces DNA breaks by free radical production. So these levels reflect the individual antioxidant status (Collins, 2014; Giovannelli et al., 2003).

This could be interpreted that the subjects in the present study had a better antioxidant defense, compared to the subjects in the investigation of Müllner et al. However there were some differences in the comet assay protocol, because in contrast to Müllner et al. we conducted the 12- gel method and we performed some additional washing steps, where as Müllner et al. performed the 2 gel method and they implemented a neutralization step after electrophoresis (Müllner et al., 2013). There are as well some modifications in the comet assay protocol, which influence results and therefore lead to different outcomes. This can be due to different concentrations or volume of agarose, different amounts of cell suspensions, differences in the period of alkaline incubation prior to electrophoresis and many more. So the comet assay has some theoretical issues to consider, and baseline data from different labs cannot really be compared but it is a useful method for evaluating DNA damage in human cells (Collins, 2014).

4.3 Correlation between biochemical and anthropometric parameters and DNA damage

In the present study no significant correlations of DNA damage with parameters such as HbA1c, Insulin, duration of T2DM or age were found. Further no significant correlations between DNA damage and any other parameters such as BMI or biochemical parameters like iron, c-peptide, HDL, LDL, cholesterol, or others were observed. On the contrary Collins et al. found a correlation of DNA damage and BMI in patients with T1DM (Collins et al., 1998). In term of HbA1c there are results which are in agreement with other finding, when patients with similar HbA1c were compared (Ibarra-Costilla et al., 2010). However, previous studies have reported positive correlations between DNA damage parameter and HbA1c in T2DM patients. This was shown for 32 subjects with a mean HbA1c of 7.7 ± 1.7 % (Tatsch et al., 2012). In terms of age an association with DNA damage has been demonstrated in the past (Piperakis et al., 1998). But on the other hand no association was found of age with damage to DNA in diabetics. Maybe the strong effect of the disease itself lead to a lack of significant impacts for age (Ibarra-Costilla et al., 2010). In our investigation we could

find a weak correlation (r= 0.175; p < 0.005) between blood glucose level and DNA strand breaks (lysis) in whole blood (figure 6).

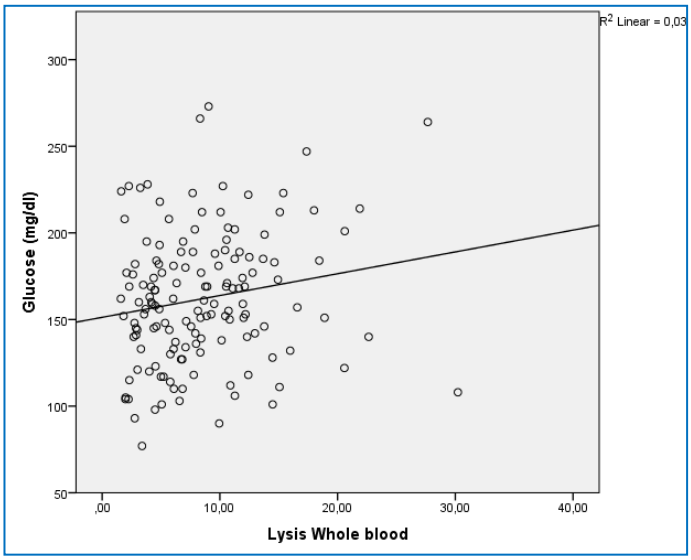

Figure 6: Correlation between glucose level and DNA damage (lysis) in whole blood (r= 0.175; p < 0.05)

On the other hand we found also some conflicting correlations. A negative correlation (r=-0.299; p=0.001) between levels of DNA strand breaks (lysis) in PBMC'S and DNA strand breaks (lysis) in whole blood was observed (figure 7).

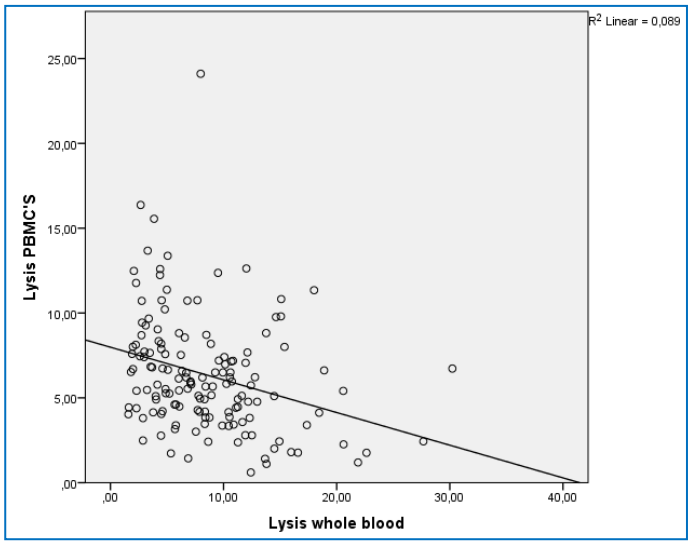

Figure 7: Correlation between DNA strand breaks (lysis) in PBMC'S and whole blood (r= -0.299; p=0.001)

A possible explanation for differences may be due the fact that the visual scoring of DNA damage was conducted by two different person. So one person was counting PBMC'S and another person was counting the DNA damage in whole blood. Forchhammer et al. have reported that the visual scoring is user dependentand that this can be seen as a determining factor (Forchhammer et al., 2008). Furthermore as already mentioned, Collins et al. reported that there are some difficulties in comparing DNA damage in isolated lymphocytes and whole blood (Collins et al., 2014).

4.4 HbA1c and DNA damage

HbA1c is the best marker for chronic hyperglycemia, since it reflects the average of the blood glucose level over a period of two to three months. For patients with diabetes, HbA1c plays an important role to manage their disease (ADA, 2014). The first aim of the present study was to examine the relationship between HbA1c and DNA damage in female T2DM patients. Therefore all subjects were divided into two subgroups, into the good glycaemic control (HbA1c ≤ 7.5 %) and the poor glycaemic control (HbA1c > 7,5 %). 74 subjects were well controlled and 72 were poorly controlled. But our measurements did not show any significant differences in DNA strand breaks, FPG-

sensitive sites or H_2O_2 induced DNA damage, neither in PBMC'S nor in whole blood (figure 8).

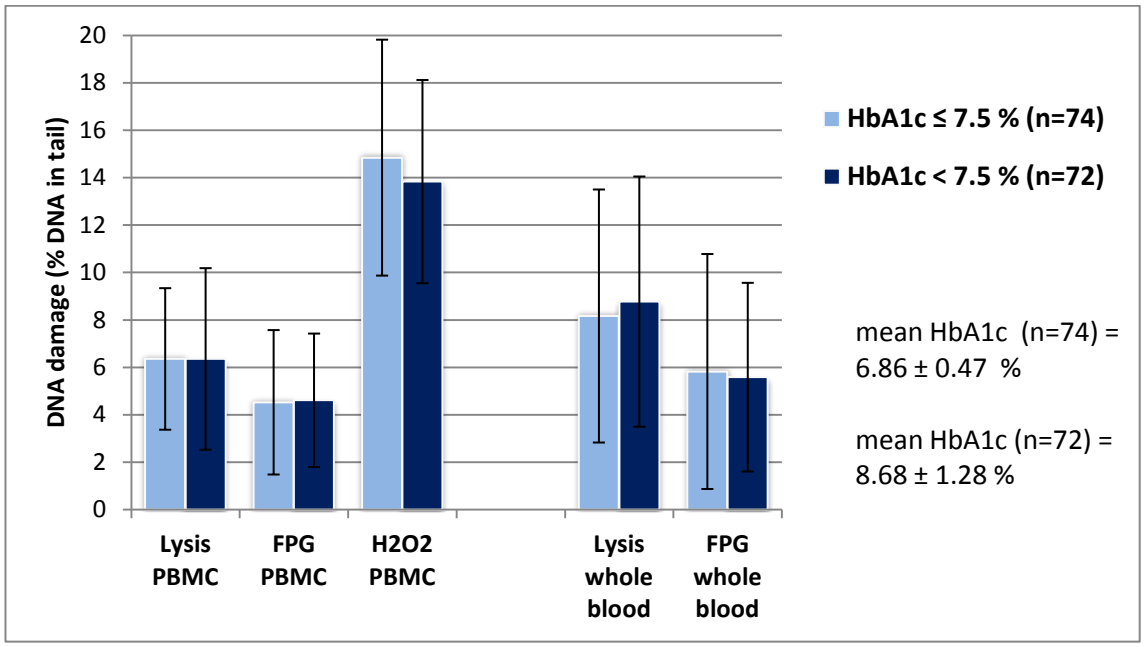

Figure 8: DNA damage in subjects with high vs. low HbA1c (Mann-Whitney U Test)

Contrary to our findings Xavier et.al. reported in their investigation in subjects with T2DM, that DNA damage was significantly ($p < 0.05$) higher in patients with a very high HbA1c (10.1 ± 1.2 %) then in patients with a lower HbA1c (6.7 ± 0.9 %) (Xavier et al., 2015). Dinçer et al. also reported from a significantly higher DNA damage in FPG sensitive sides in diabetics which were poorly controlled (n=15), compared to good controlled ones (n=18). But in their investigation there was a larger difference in the good controlled diabetics with a mean HbA1c of 5.7 ± 0.4 %, and the poorly controlled diabetics with a mean HbA1c of 9.4 ± 1.9 % (Dincer et al., 2002). Ibarra-Costilla et al. divided diabetics in three subgroups, which were all quite similar (HbA1c=10.43 ± 1.61 %, HbA1c=9.87 ± 1.64 %, and HbA1c=8.88 ± 1.35 %). No significant link between HbA1c and damage to DNA was found (Ibarra-Costilla et al., 2010). The same was observed in this study, no significant correlation between DNA damage and HbA1c. Even if there are variations in the HbA1c level of our subjects the study population may still be to homogenous for evaluation of differences in DNA damage. The hypothesis that the quality of HbA1c influences DNA damage in patients with T2DM has to be rejected in

our examination. However, when comparing healthy controls with T2DM patients differences were reported. In leukocytes from subjects with T2DM a significantly higher levels of oxidative DNA damage was shown when diabetics were compared to healthy individuals, and a positive association was shown between HbA1c level and antioxidant capacity in the group of diabetics which were poorly controlled (Lodovici et al., 2008). Furthermore, Tatsch et al. compared T2DM with a mean HbA1c of 7.7 ± 1.7 % to healthy subjects with a HbA1c of 5.2 ± 0.7 %. They found a significant correlation ($r = 0.575$; $p < 0.001$) between HbA1c and DNA damage (Tatsch et al., 2012).

4.5 Blood glucose level and DNA damage

Blood glucose or blood sugar levels are literally the amount of glucose in the blood and are measured at a single point. So this is a short-term marker (Diabetes.co.uk, 2015). An increase in blood sugar levels (hyperglycemia) is the result from an impaired insulin secretion and/or insulin action (ADA, 2003). Higher fasting blood glucose in T2DM patients is associated with higher DNA damage (Tatsch et al., 2012). Xavier et al. investigated the dietary and medically impacts on blood glucose and DNA damage. They conducted a seven-day hospitalization period in ten patients with T2DM in order to achieve adequate blood glucose levels by a dietary intervention in combination with medication treatment. This was compared to sixteen non-diabetic individuals. All subjects received a special diet with low sugar levels. Comet assay was conducted and the hOGG1 enzyme was used to evaluate oxidative DNA damage (8-OHdG). This seven-day period at the hospital with the aim of improving glycaemic control had a significant ($p < 0.05$) effect on DNA damage in peripheral blood cells from patients with T2DM. Compared to levels of DNA damage in controls, diabetics still showed higher DNA damage (D. J. Xavier et al., 2014). Moreover a link between blood glucose in diabetics and increased oxidative stress was reported too (Gelaleti et al., 2015). Hence we investigated the impact of blood glucose on DNA damage. Subjects were divided into tertiles relating to their blood glucose concentration. While group one (n=49) included the lowest levels of glucose concentration, with a mean glucose level of 122 ± 17 mg/dl, group two (n=48) with a mean blood glucose of 160 ± 8.8 mg/dl, and group three (n=49) included the highest concentrations, with a mean glucose level of 204 ±

24 mg/dl. In terms of DNA strand breaks (lysis), and H_2O_2 induced DNA damage, no significant outcomes were found. But there was a tendency towards FPG sensitive sides in whole blood (p = 0.057) and blood glucose level, when group two (mean % DNA in tail = 4.77 ± 4.36 %) was compared to group three (mean % DNA in tail = 6.54 ± 4.67 %) (Figure 9). Further there was a tendency (p = 0.056) in FPG sensitive sites and blood glucose level in PBMC'S when group one (mean % DNA in tail = 4.25 ± 2.65 %) was compared to group three (mean % DNA in tail = 5.30 ± 2.75 %) (Figure 9). So there was a tendency towards higher DNA damage related to FPG sensitive sites in subjects with higher blood glucose levels, however, this was not statistically significant.

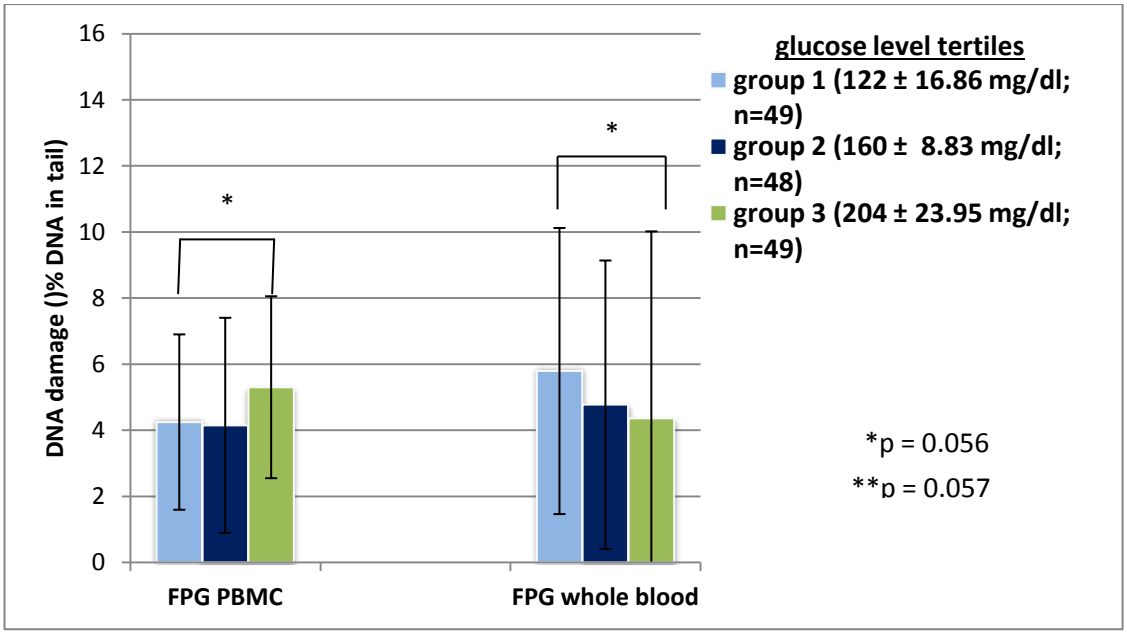

Figure 9: FPG sensitive sites (whole blood) in three groups related to blood glucose level (Independent-Samples T Test)

Collins et al. reported previously that FPG is a good marker for oxidative stress, because it reflects especially the damage in DNA that is resulting from hyperglycemia, particularly 8-oxo-guanine (Collins et al., 1998). Furthermore many studies reported from a correlation between FGP sensitive sites and blood glucose levels in diabetics (Collins et al., 1998; Dincer et al., 2002; Tatsch et al., 2012). In our investigation the

correlation between FPG sensitive sites and blood glucose in PBMC'S was 0.157. So there was a positive correlation, however it was not statistically significant.

4.6 Diabetes duration and DNA damage

To prove the secondary hypothesis whether diabetes duration of subjects influences DNA damage, subjects were again divided into tertiles relating to diabetes duration. Group one (n=49) had a mean diabetes duration of 6.94 ± 3.09 years, group two (n=48) 13.35 ± 1.14 years, and group three (n=49) 22.96 ± 7.35 years. In non of the treatments (Lysis, FPG, H_2O_2) there was any statistical significance in DNA damage, neither in PBMC'S nor in whole blood (figure 10). So we can conclude that there were no differences in DNA damage with regard to the duration of T2DM. Therefore the secondary hypothesis has to be rejected too.

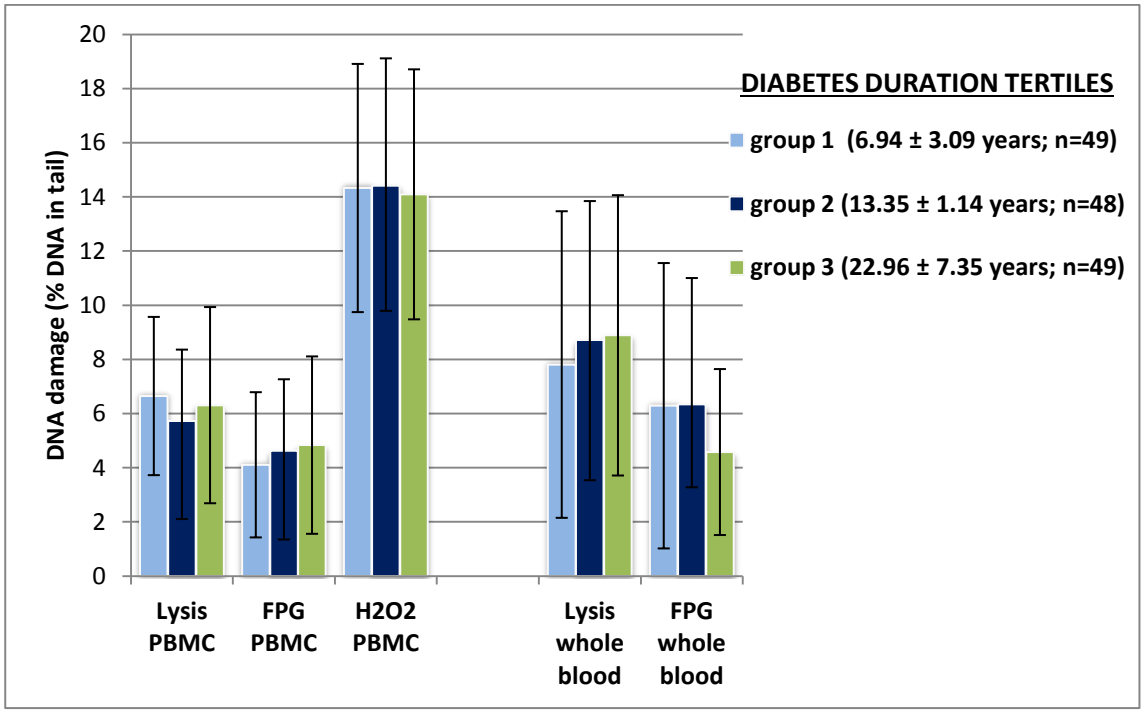

Figure 10: DNA damage due to diabetes duration (Mann-Whitney U test)

Other studies were publised, where the effect of diabetes duration on DNA damage has been investigated. Ibarra-Costilla et al. investigated the effect of long-term diabetes on DNA damage and compared subjects to healthy controls with a similar BMI. T2DM group one had a mean diabetes duration of 9.45 ± 3.91 years, T2DM group

two had a mean diabetes duration of 10.47 ± 3.82 years, and T2DM group three had a mean diabetes duration of 15.23 ± 6.05 years. They reported that the damage to DNA does not differ between subjects with long-term T2DM and healthy controls. As a possible explanation they mentioned that cells from both groups, diabetics and controls, undergo similar oxidative damage on a regular basis (Ibarra-Costilla et al., 2010). Tyrberg et al. investigated that OGG1, which is known to be involved in repair mechanisms of oxidative damage to DNA, is up-regulated in the islets of patients with T2DM. The intensity of OGG1 expression, is directly correlated with the diabetes duration. They discussed that this upregulation is a response to the increased levels of 8-OH-dG, and that long-term exposure may be relevant for the up-regulation of OGG1. The specific elevation of OGG1 in the islet cell is consistent with the idea that an increased β-cell mitochondrial oxidative metabolism which is due to hyperglycemia, may be a major factor behind the damage to DNA. The exact mechanism by which OGG1 protein is upregulated in diabetic islets is so far unknown (Tyrberg et al., 2002). Colak et al. reported from a significantly negative correlation SOD and total antioxidant defense with diabetes duration. This was around in a group of subjects with T2DM which suffered additionally from coronary artery disease and hypertension (Colak et al., 2005).

4.7 Age and DNA damage

In general it is assumed that damage to DNA accumulates with increasing age. Hence higher levels of strand breaks and/or oxidized bases are expected in higher age. An increase in DNA damage is shown in some studies (Dusinska and Collins, 2008; Moller, 2006; Staruchova et al., 2008). However results are not always consistent (Tatsch et al., 2012). For measuring the age related DNA damage in our study population we divided subjects into three age-groups. Group one from 40-62 years (n=40) group two from 63-73 years (n=61) and group three 74 and older (n=45). We did not find any significant impact of age on DNA damage in PBMC'S or whole blood, in DNA strand breaks and FPG sensitive sides. But a significant difference was observed in H_2O_2 induced DNA damage, when group one was compared to group three (p=0.018) (figure 11).

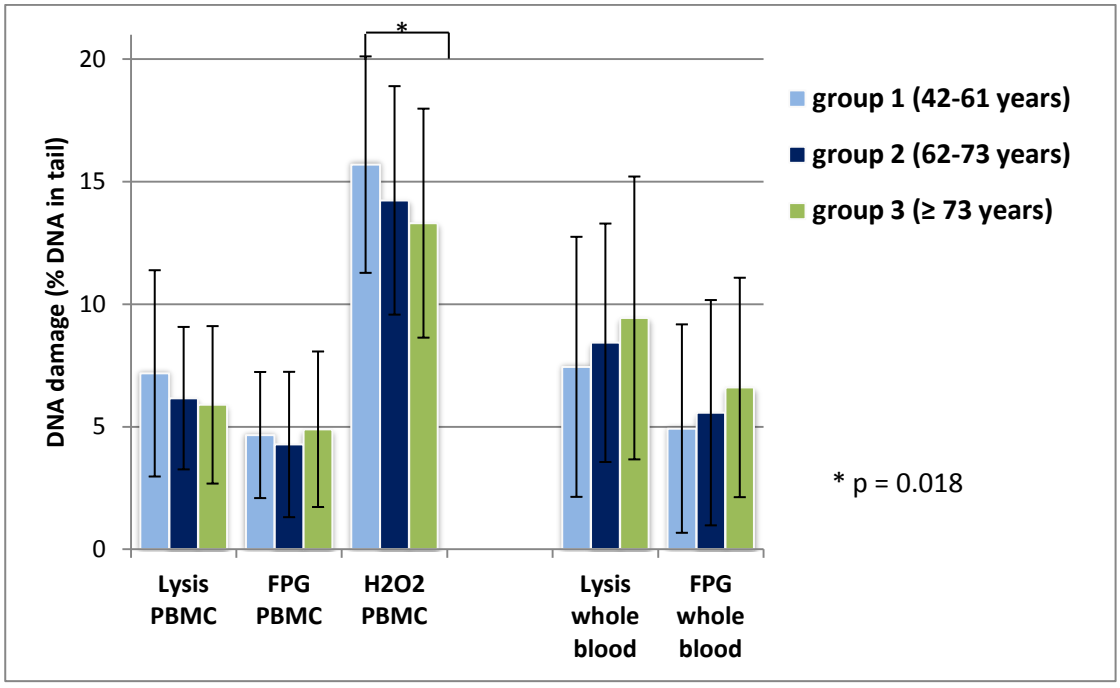

Figure 11: Mean DNA damage related to age groups (Independent-Samples T Test)

Our findings are in agreement with Tatsch et al., since they did not find any association between DNA strand breaks and age in 32 diabetic subjects (Tatsch et al., 2012). Ibarra-Costilla et al. reported from a weak increase in damage to DNA in diabetics when two younger groups (40-60 years) were compared to an older group of subjects (61-70 years). However these findings were not statistically significant. The lack of age related effects on DNA damage observed in subjects with diabetes is probably due to the strong effect from the disease itself (Ibarra-Costilla et al., 2010).

4.8 Medical treatment and DNA damage

The impact on DNA damage in diabetics due to insulin was evaluated previously (Collins et al., 2014; Tatsch et al., 2012). Trials had their focus on SU, a group of oral antidiabetics (Sawada et al., 2008) or metformin (Algire et al., 2012). Furthermore there are various studies investigated the association between cancer and diabetes with regard to insulin (Colhoun, 2009; Currie et al., 2009) and also metfromin (Currie et al., 2009; Libby et al., 2009). We focused on treatments of insulin, SU und metformin, because most of our subjects used at least one of these medications (table 8).

Table 8: Medical treatment	
Insulin (n)	60
Sulfonylurea (n)	38
Insulin + sulfonylurea (n)	94
Metformin (n)	113
Glinide	6
Acarbose	2
Alogliptin	77
GLP 1-4 (glucagon like protein)	14
Alogliptin+Metformin	64
Pioglitazon	17
Sglt (sodium-glucose cotransporter)	16

4.8.1 Insulin treatment and DNA damage

Individuals with T2DM have an insulin resistance and usually they have a relative insulin deficiency, rather than absolute. At the onset of the DM disease insulin treatment is not necessary. Some individuals never need any insulin treatment for daily live, but others do need insulin to lower blood glucose (ADA, 2014). In our study 60 subjects required insulin treatment. To evaluate the link between insulin treatment and DNA damage subjects were divided into two groups, one group of subjects which were treated with insulin (n=60) and one group of subject with other treatments (n=86). We found significant outcomes in terms of insulin treatment and DNA strand breaks (lysis) of PBMC'S and in H_2O_2 induced DNA damage in PBMC'S (figure 12). DNA damage in subjects without insulin treatment was higher than to those with insulin treatment. The mean % DNA in tail in subjects with insulin treatment was 5.67 ± 3.32 %. and without insulin treatment 6.83 ± 3.42 % (p= 0.014). DNA damage due to H_2O_2 challenge was also significantly different. The mean % DNA in tail in subjects with insulin treatment was 13.14 ± 4.84 % and without insulin treatment 15.19 ± 4.36 % (p =

0.005). A lower level of H_2O_2 induced DNA damage is associated with a better antioxidant defense (Collins, 2014).

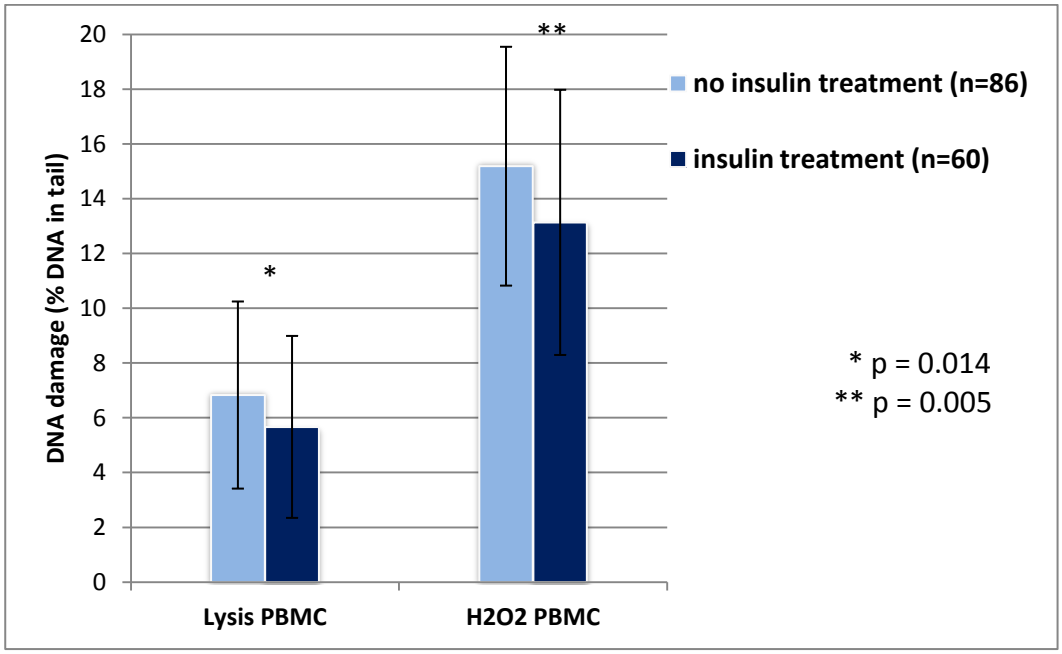

Figure 12: DNA strand breaks and H_2O_2 induced DNA damage in T2DM subjects, which were treated with or without insulin (Mann-Whitney U test)

Tatsch et al. did not observe any association between DNA tail damage and insulin treatment on 32 DM subjects (Tatsch et al., 2012). However, the impact of insulin treatment on 33 diabetics was investigated by Seghrouchni et al. They observed that oxidative stress parameters of insulin treated T2DM patients were in between of insulin depended diabetes mellitus (IDDM) and non insulin depended diabetes mellitus (NIDDM). They separated insulin treated T2DM and IDDM into groups, according to the duration of their insulin treatment (< 10 years and ≥ 10 years). All four groups had a similar HbA1c. They found slightly lower oxidative stress parameters in T2DM when insulin treatment was longer. Hence long insulin treatment can improve some of the oxidative stress parameters for insulin treated T2DM (Seghrouchni et al., 2002). And oxidative stress is probably the main mediator in damage of DNA in diabetics (Lee and Chan, 2015). Nevertheless in terms of insulin there are some outcomes, which indicate that insulin treatment is associated with higher risk of cancer, like liver or pancreatic

cancer (Currie et al., 2009; Schlesinger et al., 2013). In contrast many other outcomes did not find any association with an increased cancer risk (Colhoun, 2009; Mannucci et al., 2010; Simó et al., 2013) In our investigation DNA damage in PBMC'S was lower in subjects with insulin treatment, therefore we can conclude that in our subjects insulin treatment is associated with lower DNA damage.

4.8.2 Sulfonylurea and DNA damage

38 subjects in our study used SU as oral antidiabetic drug. SU lowers blood sugar level by increasing the release of insulin by blocking potassium-channel in the beta cells of the pancreas (Matthaei S. and G. Schernthaner, 2009). Sawada et al. investigated the effects of SU, a group of oral antidiabetics. They reported that some SU (glimepiride and glibenclamide, but not gliclazide) and also nateglinide (a drug for T2DM treatment) induced the production of ROS in vitro. Due to the PKC–dependent activation of the NAD(P)H oxidase, *β*-cell apoptosis is caused consequently. Even though SU are commonly used for treating T2DM, there is concern that constant use of SU may lead to dysfunction of the *β*-cell and apoptosis (Sawada et al., 2008). Subject in our study were divided into two groups, in those, which were treated with SU (n=38) and those without SU treatment but with other medications (n=107). There was no significant link to DNA damage in any treatment (H_2O_2, Lysis, FPG,) or type of cell (PBMC'S or whole blood) in our investigation (figure 13). Thus we can conclude that there was no higher DNA damage due to SU treatment in subjects with T2DM.

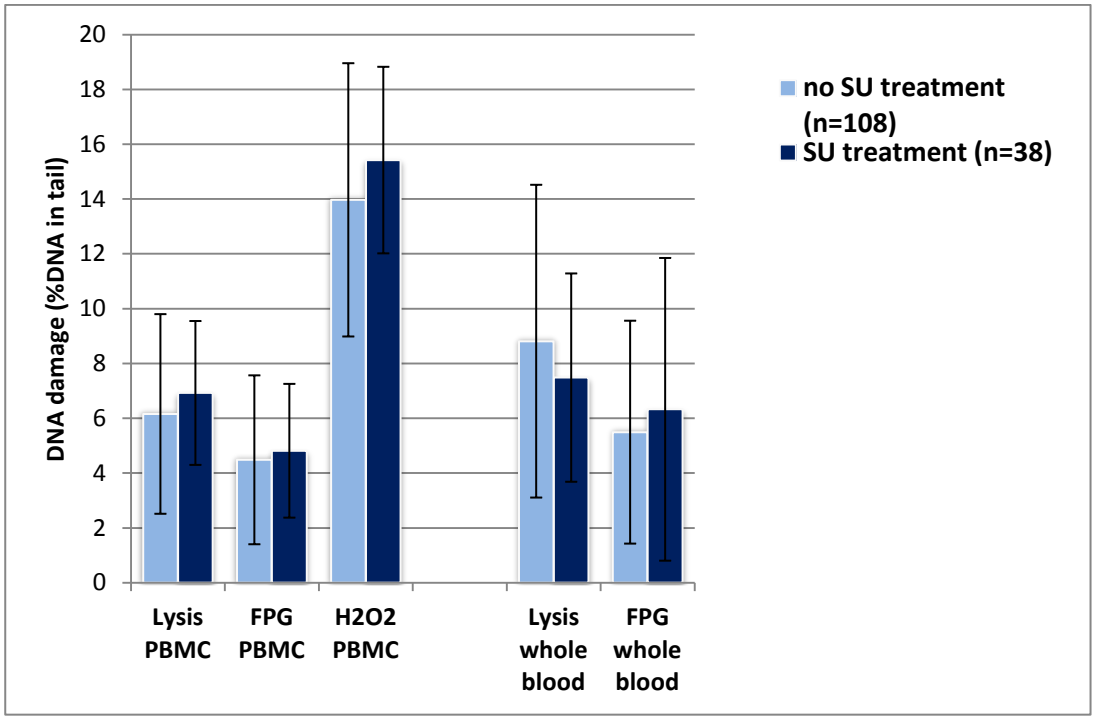

Figure 13: DNA damage in subjects with T2DM, which were treated with or without sulfonylurea (Independent-Samples T Test)

4.8.3 Insulin and/or sulfonylurea and DNA damage

To determine whether there is a link between DNA damage and insulin and/or SU treatment, these two variables were combined. Hence subjects with insulin and/or SU as treatment (n=94) were compared to the remaining subject (n=52). Here again, no significant outcomes in DNA damage, regardless the treatment, neither in whole blood nor in PBMC'S was observed (figure 14). The differences seen for insulin was no longer apparent.

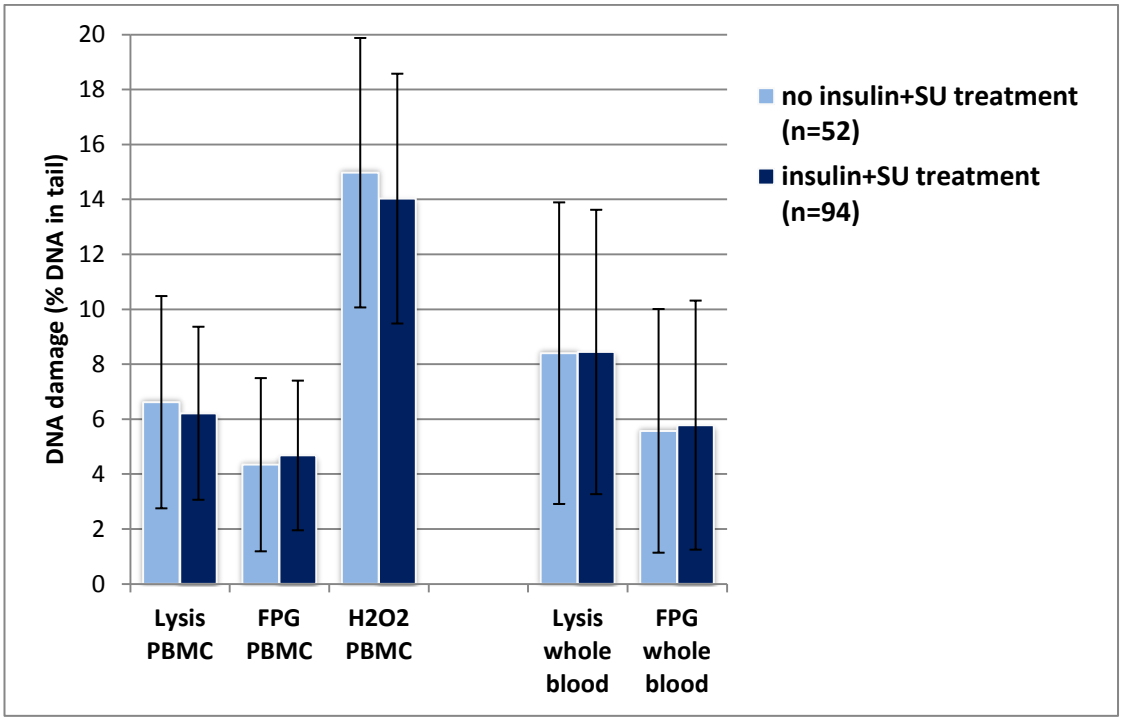

Figure 14: Mean DNA damage in subjects with or without insulin and/or sulfonylurea treatment (Mann-Whitney U test)

Alvarsson et al. conducted a prospective study to investigate the effects of insulin treatment versus SU treatment in patients with T2DM. They found that initial insulin treatment in T2DM showed a better outcome in terms of endogenous insulin secretion and also of the metabolic control compared to a conventional SU (glibenclamide) treatment (Alvarsson et al., 2003).

Sulfonylurea without the influence of insulin treatment

We excluded all patients, which were treated with insulin and tested the effect of SU on DNA damage again. 52 subjects without insulin and SU treatment were compared to 34 subjects with SU treatment. Here were also no significant outcomes seen, regardless which cell type.

Insulin without the influence of sulfonylurea treatment

Then we excluded all subjects, which were under SU treatment and tested again if insulin treatment has an effect on DNA damage. For that 52 subjects without insulin and SU treatment were compared to 56 subjects, which were under insulin treatment. Again there was a significant outcome ($p = 0.034$) in terms of H_2O_2 induced DNA damage and insulin treatment. The mean % DNA in tail was 14.92 ± 4.84 % in subjects without insulin treatment and 13.08 ± 4.99 % in subjects with insulin treatment after H_2O_2 challenge.

4.8.4 Metformin and DNA damage

Metformin, an oral antihyperglycemic drug, is supposed to be the first choice of therapy when tolerated (Salvatore et al.). In our cross-sectional study most subjects (n=113) were treated with metformin. So we evaluated the effect of metformin on DNA damage. However there were no significant link to DNA damage in PBMC'S or whole blood found, regardless which treatment (Lysis, FPG, H_2O_2), when subjects with metformin treatment (n=113) were compared to those without metformin treatment (n=33) (figure 15). Then we excluded all patients, which were treated with SU or insulin. Just a small number of subjects remained. Six subjects were not treated with metformin and 28 were treated with metformin. Also for this testing no difference in DNA damage was found in any type of cell. Therefore we can conclude that metformin has no effect on DNA damage in T2DM patients in our study.

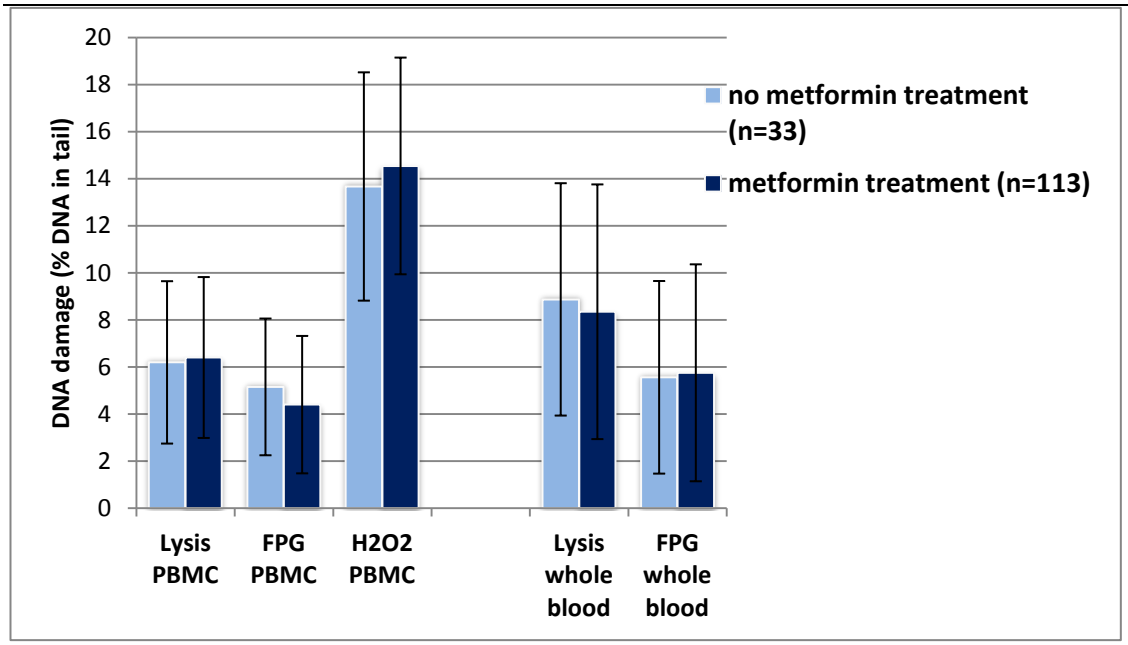

Figure 15: Metformin treatment and DNA damage (Independent-Samples T Test)

Metformin has the potential to increases insulin sensitivity and to decreases hyperinsulinemia, and metformin is also associated with a lower incidence of cancer in patients with diabetes than in those, which were treated with insulin or SU (Currie et al., 2009; Libby et al., 2009). Nevertheless T2DM is also associated with an increased risk in various types of cancer (Shikata et al., 2013). So there is particular interest in the potential of drugs, which may reduce the cancer risk, therefore various studies have been conducted to investigate the impact of metformin. Libby et al. conducted an observational cohort study in T2DM patient with novel diagnosed cancer. They reported from a significantly reduced risk of cancer in subjects which were treated with metformin compared to those without metformin treatment (Libby et al., 2009). There is increasing evidence that metformin inhibits the growth of cancer cells in vitro and also the tumor proliferation in animals (Ben Sahra et al., 2010). Even if the data for pancreatic cancer are controversial, Wang et al. reported from the indication that metformin decreases the risk of pancreatic cancer in patients with T2DM. They concluded also that further studies are needed to investigate the effects of metformin in chemoprevention of T2DM patients with pancreatic cancer and also its antitumor activity (Wang et al., 2014). Furthermore Algier et al. reported that metformin

can reduce endogenous ROS production, oxidative stress, DNA damage and it can reduce mutagenesis in normal somatic cells. They conclude that metformin may provide a new mechanism for the cancer risk reduction (Algire et al., 2012). Onaran et al. concluded that metformin at pharmacological concentrations had no potential to modify chemically induced DNA damage in cultured human lymphocytes, but on lipid peroxidation it was partly protective (Onaran et al., 2006).

4.8.5 Metformin vs. insulin and DNA damage

In our cross-sectional study 60 subjects were treated with insulin and 113 with metformin. For insulin treatment we found a significantly lower DNA damage in H_2O_2 sensitive sites ($p = 0.005$) and in DNA strand breaks (lysis) ($p = 0.014$) when subjects were treated with insulin compared to subjects without insulin treatment. In terms of metformin we did not find any association between the treatment and DNA damage. In table 10 we evaluated the mean DNA damage in subjects with insulin treatment (60) and compared this numbers to the mean DNA damage in subjects with metformin treatment (113). There is a slightly but insignificantly higher DNA damage in all treatments, apart from FPG in PBMC'S, in subjects, which were treated with metformin.

Table 9: DNA damage (%DNA in tail) in PBMC'S and whole blood of subjects with insulin vs. metformin treatment

	Mean ± SD % DNA in tail from Insulin (n=60)	**Mean ± SD % DNA in tail from metformin (n=113)**
PBMC'S:		
Lysis:	5.67 ± 3.32 %	6.40 ± 3.42 %
H_2O_2:	13.18 ± 4.84 %	14.54 ± 4.61%
FPG:	4.48 ± 2.96 %	4.40 ± 2.12%
Whole blood:		
Lysis:	9.23 ± 5.82 %	8.35 ± 5.41%
FPG:	5.40 ± 3.7 %	5.75 ± 4.61%

4. Metabolic syndrome and DNA damage

Due to the fact that almost all of our subjects are suffering from MetS we evaluated whether MetS in correlated to DNA damage. MetS is a worldwide health problem, and its pathogenesis is not yet clear. Karaman et al. evaluated the effects of MetS on DNA damage via the comet assay. Fifty-two patients with metabolic syndrome and 35 healthy controls were evaluated. The comet tails length was significantly higher in the patients with MetS ($p < 0.001$) (Karaman et al., 2015). Mili´c et al. investigated DNA damage in the early stage of MetS including 56 healthy controls compared to 65 subjects with MetS. In this study no differences in DNA damage between MetS and healthy controls were observed. Additionally they reported that the level of 8-oxo-dG was higher in controls than in patients with the MetS. In the early stage of Mets no differences to controls were found (Milic et al., 2015). Kamaran et al. evaluated enzyme activities to draw a conclusion about oxidative stress and MetS. SOD and GPx enzyme activities were significantly lower in the group with MetS. They concluded that MetS is associated with increase in DNA damage and oxidative stress (Karaman et al., 2015). Demirbag et al. showed that DNA damage which was assessed with the alkaline comet assay, is increased in subjects with MetS and that parameters of oxidative stress, such as total antioxidant capacity and total peroxides, are decreased in the group with MetS compared to controls (Demirbag et al., 2006). The considerable number of 140 subjects were suffering from the MetS in our study. We divided subjects due to the severity of Mets. We compared subjects, which met three criteria (n=51) to subjects which met all five criteria (n=31) of the metabolic syndrome, according to IDF. In terms of FPG, we did not find any differences between these two groups in PBMC and whole blood (figure 16). Further there were no differences in H_2O_2 induced DNA damage, or DNA strand breaks when these two groups were compared.

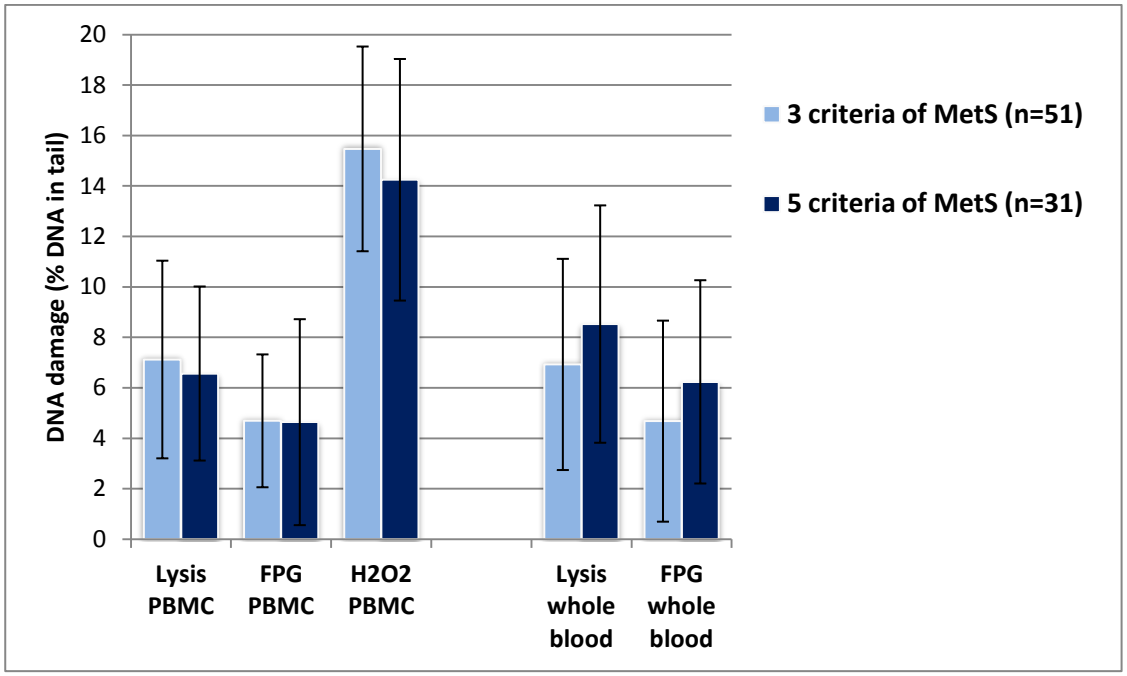

Figure 16: DNA damage of groups with three or five criteria of the metabolic syndrome (Independent-Samples T Test)

4.10 Smoking and DNA damage

Another factor that leads to increased DNA damage is smoking. Therefore we evaluated the smoking history of our subjects, and tested whether former smokers have higher DNA damage than subjects which were non-smokers for their whole life. We compared 72 non-smokers to 74 former smokers, with at least on without tobacco use. Also here we could not find any significant differences in DNA damage, regardless which treatment (Lysis, H_2O_2, FPG) neither in PBMC'S nor in whole blood (figure 17).

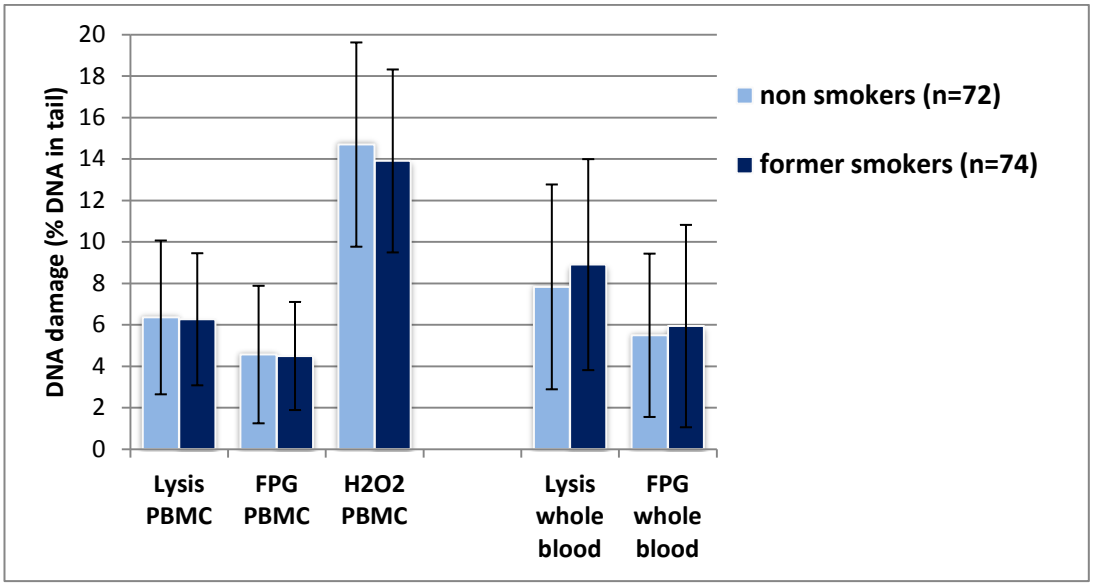

Figure 17: Smoking history and DNA damage (Independent-Samples T Test)

We are in agreement with the outcomes from Tatsch et al., who also did not find any association between smoking habits and T2DM (Tatsch et al., 2012). In previous studies conflicting results have been reported, when the impact of tobacco use on DNA damage was measured (Moller et al., 2000). Oxidative damage in lymphocytes were measured in 155 middle-aged men, a significantly higher DNA damage was found in smokers compared to non-smokers (Dusinska et al., 2001). A similar study with twelve young healthy male heavy smokers (more than 20 cigarettes per day) and twelve non-smokers was conducted in order to evaluate DNA damage. Comet assay effects in fresh whole blood samples and isolated lymphocytes showed no significant differences between young heavy smokers and controls (Hoffmann and Speit, 2005). So there are many different outcomes in terms of DNA damage and smoking habits. Moller et al. reviewed that buccal cells and nasal epithelial cells have shown effects when DNA damage with regard to smoking habits was evaluated, because these cells are from tissues with direct contact with the ingested or inhaled tobacco compounds (Moller et al., 2000). So maybe we could not find any differences, because the cells we analyzed were PBMC'S and whole blood and not buccal cells or nasal epithelial cells. Furthermore DNA damage is efficiently repaired by different cellular enzymes, and therefore its measurement gives only a snapshot of the overall level of oxidative stress

in the body (Balasubramanyam et al., 2010). Further our subjects were already non-smokers since at least two years, hence it is not surprising that we could not find any significant outcomes.

5 Conclusion

In this cross-sectional study no significant differences were observed in DNA damage when 74 well controlled T2DM subjects (HbA1c ≤ 7.5 %) were compared to 72 poorly controlled T2DM subjects (HbA1c > 7.5 %). Therefore the main hypothesis has to be rejected. Furthermore no differences were investigated in DNA damage and diabetes duration when subjects were divided into tertiles (means: 6.94 ± 3.09 years, 13.35 ± 1.14 years, 22.96 ± 7.35 years). Thus the secondary hypothesis has to be rejected too. Moreover no association was found regarding DNA damage and age, in severity of the metabolic syndrome or the smoking history. With regard to medication no damage to DNA was reported in metformin treatment and SU treatment. However some outcomes showed significant results or at least weak tendencies. For blood glucose tertiles and DNA damage a tendency towards FPG sensitive sites in whole blood ($p = 0.057$) was investigated, when group two (blood glucose = 160 ± 8.8 mg/dl; % DNA in tail = 4.77 ± 4.36 %) was compared to group three (blood glucose = 204 ± 24 mg/dl; % DNA in tail = 6.54 ± 4.67 %). And there was a tendency ($p = 0.056$) in FPG sensitive sites and blood glucose level in PBMC'S when group one (blood glucose = 122 ± 17 mg/dl; % DNA in tail = 4.25 ± 2.65 %) was compared to group three (blood glucose = 204 ± 24 mg/dl; % DNA in tail = 6.54 ± 4.67 %). So there was a tendency towards higher DNA damage related to FPG sensitive sides in subjects with higher blood sugar levels. However this was not observed with HbA1c. Furthermore there was an association between DNA strand breaks (lysis) and H_2O_2 induced DNA damage in PBMC'S and insulin treatment. A significantly higher DNA damage was observed in subjects without insulin treatment (n=86) compared to those with the need of insulin treatment (n=60). This was observed in H_2O_2 induced DNA damage (with insulin treatment: 13.14 ± 4.84 % DNA in tail; without insulin treatment: 15.19 ± 4.36 % DNA in tail; $p = 0.005$) and in DNA strand breaks (with insulin treatment: 5.67 ± 3.32 % DNA in tail; without insulin treatment 6.83 ± 3.42 % DNA in tail; $p = 0.014$). A significant difference in insulin treatment was seen, even if subjects with SU treatment were excluded. Fifty-two subjects without insulin treatment showed higher H_2O_2 induced DNA damage in PBMC'S (14.92 ± 4.84 % DNA in tail) than 56 subjects with insulin treatment (13.08 ±

4.99 % DNA in tail) ($p = 0.034$). Some conflicting results were obtained when DNA damage in PBMC'S and whole blood was compared. A significant ($p = 0.001$) negative correlation ($r = -0.299$) between levels of DNA strand breaks (lysis) in PBMC'S and DNA strand breaks (lysis) in whole blood were observed.

With the comet assay we could not observe significant differences between DNA damage and HbA1c or diabetes duration in females with T2DM. We conclude, that the study population was to homogenous, and that females with T2DM in Austria were under good medical treatment. Therefore the comet assay was maybe not sensitive enough to show significant associations in DNA damage in our investigation. Further in the future it should be considered that just one person is counting PBMC'S and whole blood, in order to avoid inconsistencies related to visual counting.

6 Summary

DM is one of the most common chronic diseases worldwide, with a continuously increasing prevalence and incidence. Worldwide 387 million people are suffering from DM, from which T2DM is representing approximately 90-95 % of all cases. DM is characterized by an increase in the blood sugar levels, the chronic hyperglycemia, which can cause DNA damage. Hyperglycemia, and its common additional existing risk factors hypertension, and dyslipidemia, are leading to a direct toxic effect of sugar on tissues, and thereupon to changes in small and large vessels, called micro- and macrovascular complications. Furthermore many studies showed evidence for an increased risk of mortality from cancer among diabetics. This evidence has been investigated in different types of cancer. Oxidative stress is the most important mediator of DNA damage in diabetics. The accumulation of DNA damage may lead to mutations. Therefore, damage to DNA is possibly an important biological link between DM and cancer.

In the present master's thesis, which was performed within the cross-sectional study "MIKRODIAB", the impact of T2DM on DNA damage was evaluated by the comet assay in 146 female subjects, which were recruited during their routine check at the Diabetes Outpatient Clinic South in Vienna. DNA strand breaks (lysis) and FPG sensitive sites were evaluated in PBMC'S and in whole blood. Furthermore H_2O_2 induced DNA damage was examined in PBMC'S. For the main hypothesis subjects were divided into two groups with well glycaemic control (HbA1c ≤ 7.5 %; n=74) and poor glycaemic control (HbA1c > 7.5; n=72) and DNA damage was evaluated. For the second hypothesis subjects were divided into tertiles related to their diabetes duration (means: 6.94 ± 3.09 years, 13.35 ± 1.14 years, 22.96 ± 7.35 years).

No significant difference in DNA damage was evaluated when well controlled T2DM subjects were compared to poorly controlled T2DM subjects. Furthermore no significant differences were obtained in DNA damage with respect to diabetes duration. Therefore the main and the secondary hypothesis have to be rejected.

We conclude, that the study population was to homogenous to detect significant differences in DNA damage and HbA1c or diabetes duration by comet assay.

7 Zusammenfassung

Diabetes mellitus ist eine der am häufigsten vorkommenden chronischen Erkrankungen mit einer kontinuierlich steigenden Prävalenz und Inzidenz. Weltweit leiden 387 Millionen Menschen an DM, wovon T2DM mit 90-95 % den Großteil der Erkrankungen ausmacht. DM ist gekennzeichnet durch einen Anstieg des Blutzuckers, der sogenannten chronischen Hyperglykämie. Die metabolischen Faktoren führen zu DNA-Schäden. Hyperglykämie und die damit verbundenen Risikofaktoren wie Bluthochdruck und Fettstoffwechselstörung führen zu einer direkt toxischen Wirkung von Zucker auf das Gewebe und somit zu Veränderungen kleiner und großer Gefäße, den mikro- und makrovaskulären Komplikationen. Viele Studien zeigen Hinweise auf ein erhöhtes Sterblichkeitsrisiko bei Diabetikern die an Krebs leiden. Diese Evidenz wurde bei verschiedenen Krebserkrankungen gezeigt. Oxidativer Stress gilt als wichtigster Mediator bei der Entstehung von DNA-Schäden bei Diabetikern. Die Akkumulation dieser DNA-Schäden kann zu Mutationen führen. Somit sind DNA-Schäden eine mögliche biologische Verbindung zwischen DM und Krebs.

In dieser Masterarbeit, welche im Rahmen der Querschnittstudie "MIKRODIAB" durchgeführt wurde, wurden die Auswirkungen von T2DM auf DNA-Schäden mittels comet assay untersucht. 146 weibliche Typ 2 Diabetikerinnen wurden innerhalb ihrer routinemäßigen Untersuchungen, in der Diabetesambulanz im Gesundheitszentrum Wien-Süd, rekrutiert. DNA Strangbrüche (Lyse) und oxidative DNA-Schäden (FPG) wurden in isolierten Lymphozyten (PBMC'S) und in Vollblut evaluiert. Weiters wurden auch H_2O_2 induzierte DNA-Schäden in PBMC'S untersucht. Um die Haupthypothese zu prüfen wurden die Probandinnen nach ihrer glykämischen Kontrolle in zwei Gruppen geteilt. 74 Probandinnen gehörten der gut kontrollierten Gruppe (HbA1c ≤ 7.5 %) an und 72 Probandinnen gehörten der schlechte kontrollierten Gruppe (HbA1c > 7.5) an. DNA-Schäden wurden mittels Comet Assay evaluiert. Für die zweite Hypothese wurden die Probandinnen in Tertilen geteilt, welche nach Diabetesdauer eingeteilt waren (Mittelwerte: 6.94 ± 3.09 Jahre, 13.35 ± 1.14 Jahre, 22.96 ± 7.35 Jahre). Es wurden keine signifikanten Ergebnisse in DNA-Schäden und gut kontrollierten Typ 2 Diabetikerinnen versus schlecht kontrollierter Typ 2 Diabetikerinnern erhalten. Weiters

wurden auch keine Verbindungen zwischen DNA-Schäden und der Diabetesdauer evaluiert. Daher müssen die Haupthypothese und auch die zweite Hypothese verworfen werden.

Abschließend ist zu sagen, dass keine Unterschiede in DNA-Schäden unter Berücksichtigung von HbA1c oder der Diabetesdauer bei Typ 2 Diabetikerinnen in Österreich evaluiert werden konnte. Wir schließen daraus, die Studienpopulation zu homogen war um signifikante Unterschiede in DNA-Schäden mit dem Comet Assay zu erhalten.

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Appendix

Chemicals and working equipment

Materials/Equipment	Producers	Product number
Analytical balance 0,01 mg-220g	Mettler	AT 201
Centrifuge	Eppendorf	5417R
Countess, automated cell counter	Invitrogen	C10227
Countess cell counting chamber slide	Invitrogen	C10283
Coverslip 24 x 32	VWR	631-1572
Electrophoresis Power Supply	PeqLab	EV231
Electrophoresis chamber	PeqLab	41-2325-R
Fluorescence microscope (20x/0,40)	Nikon	Eclipse Ci-L
Incubator	Memmert	
Lamina Flow	Euro Clone Bioair	S@FEMATE 1.8
Leucosep tubes 50 ml	Greiner Bio-one	227288
Lumen 200 (light for Fluorescence microscope)	Prior	L200D
Magnetic stirrer	Heidolph	MR 3001 K
Megafuge 1.0R	Thermo	40768330
Megafuge 40	Thermo	
Microscope slides	VWR	ECN 631-1551
Microwave	Elta	

Multi-pipette		
pH Meter	Metrohm	827 pH lab
Pipette 5 µl – 50 µl	Biohit	8103949
Pipette 20 µl – 200 µl	VWR	259050316
Pipette 100 µl – 1000 µl	VWR	259060722
Potassium Chloride (KCL)	Merck	49.360.500
Scale	Sartourius	LC 480 1P-OCE
Vortex	Heidolph	REAX 2000
Waterbath	GFL	89585

Chemicals/Reagents	**Producers**	**Product number**
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid(Hepes)(minimum 99,5% titration)	Sigma Life Science	7365-49-9
Bovine Serum Albumin (BSA)	Sigma Life Science	A 2153-50G
Dimethylsulfoxide >99,5% (DMSO)	Sigma Life Science	67-68-5
Dulbecco`s Phosphate buffered Saline (PBS)	Sigma Life Science	D 8537
Ethanol	Merck	603-002-00-5
EthylenediamineTetraacetic acid (EDTA)	Sigma Life Science	60-00-4
Fetal Bovine Serum (FBS)	PAA- The cell culture company	A15-101
Formamidopyrimidin-DNS-Glycosylase (FPG)	Biolabs	0061405
GelRed Nucleic Acid Stain	Biotium	41003

Potassium Chloride (KCL)	Merck	49.360.500
Potassium Hydroxid>86% (KOH)	Sigma Life Science	60370
Sodium Chloride (NaCl)	Sigma Life Science	7647-14-5
Sodium hydroxide pellets (NaOH)	Sigma Life Science	1310-73-2
Triton-X-100	Sigma Life Science	9002-93-1
Trizma-base	Sigma Life Science	77-86-1
Trypan blue stain 0,4%	Invitrogen	761866
Ultra Pure Agarose (NMA)	invitrogen	16500-100
Ultra Pure LMP Agaraose (low melting point)	invitrogen	16520-050

I. Preparation of general solutions and reagents

All solutions were kept in the fridge at 4°C or cooled down to 4°C before usage.

Lysis solution (pH = 10)

amounts per liter

2.5 M NaCl 146.1 g

0.1 M EDTA 37.2 g

10 mMTris 1.2 g

The pH was adjusted to 10 by using 10 M NaOH. Before use 1 ml Triton X-100 per 100 ml lyses solution was added and mixed well.

H_2O_2 Stock solution

Into 10 ml aqua bidest 103 µl conc. H_2O_2 solution was added. For H_2O_2 treatment a 100 µM solution was used.

Enzyme reaction buffer for FPG, Stock solution, (Puffer F)

amounts per 2 liter

40 mM HEPES 190.60 g

0.1 M KCl 149.12 g

0.5 mM EDTA 3.00 g

0.2 mg/ml BSA 4.00 g

With 1 M KOH the pH was set to 8.00. Aliquots were stored at -20°C. Prior to use it got melted and was diluted (1:10) with aqua bidest. And pH was measured again and adjusted to 8 by KOH once more.

Electrophoresis buffer (pH > 13)

amounts per 2 liter

0.3 M NaOH 24.00 g

0.001 M EDTA 0.58 g

Low melting agarose (LMA)

1000 mg LMA in 1000 ml PBS

This was mixed and solved in the microwave, then aliqoted and stored at 4°C.

Normal melting agarose (NMA)

1000 mg NMA in 100 ml aqua bidest

This was mixed and solved in the microwave.

GelRed

3 µl GelRed stock solution up to 10 ml aqua bidest (3:10000)

II. Comet Assay procedure (12 gel method)

Slide preparation:

- Dissolve 200 mg NMP agarose in 20 ml double distilled water by heating slowly in the microwave
- place agarose in the waterbath (55°C)
- dip slides for 2 sec. in the agarose
- wipe off the backside of the slide
- lay the wiped-off side of the slide down on a paper towel
- dry overnight (room temperature)
- place coated slides in a box, store at room temperature

Washing lymphocytes:

- prepare a small box with ice for the samples
- take samples out of the freezer at -80°C (12 samples per run)
  thaw them quickly in your hands or in a waterbath (37°C)
- centrifuge: 3000 rpm for 3 minutes at 4°C
- all following steps need to be done on ice, and rapidly
- remove supernatant
- add 1 ml of PBS
- solve by tapping the cup
- centrifuge: 3000 rpm for 3 minutes at 4°C
- remove supernatant
- add 300 µl of PBS
- solve by tapping the cup
- determine the cell amount by the countess (Invitrogen)
  mix 10 µl cell suspension and 10 µl of trypan blue, transfer 10 µl into a chamber slide

  note living cells, death cells and viability

- calculate a concentration of 6 x 10^5 living cells for each subject
  if cell amount is higher: add the calculated volume of PBS

if cell amount is lesser: centrifuge again, add the calculated volume of PBS

Prepare

- a template for labeling slides (word document)
- label slides (NMA coated slides) with PBMC'S or whole blood, the treatment (Lysis, H_2O_2, FPG, buffer) and subject numbers (use shortcuts)
- a small box with ice for the samples (place samples in a priority order)
- a large box with ice
- mix 100 µl H_2O_2 stock-solution with 100 ml of double distilled water (100 µM)
  fill it in a cuvette, place it on ice (large box)
- add 1 ml of Tritox X to 100 ml of double distilled water
  place it on the magnetic stirrer (3-5 min.)
  fill the solution in a cuvette, place it on ice (large box)
- get PBS out of the fridge, fill it in a cuvette, place it on ice (large box)
- heat LMA slowely in the microwave
  place it in the waterbath (37°C)
- 8 metal plates
- for the procedure with PBMC'S: place 4 metal plates on ice (large box)
- for the procedure with whole blood: place 3 metal plates on ice (large box)
  put rubber gloves under the metal plates (otherwise plates get moist quickly)
- multi-pipette, 2 pipettes (20-200 µl and 5-50 µl)
- for whole blood**:** take samples out of the freezer (-80°C), thaw them quickly in your hands; place samples on ice (in a priority order)

Spreading cells on slides

- place labeled slides on the metal plates
- the following steps need to be done rapidly
- **for PBMC'S:** mix 15 µl of cell suspension with 70 µl of LMA

bring cells on slides with a multi-pipette (8 times 15µl)

- **for whole blood:** mix 10 µl of whole blood with 100 µl of LMA
  bring cells on slides with a multi-pipette (6 times 5 µl)
- change metal plates for the next run

Treatment with H_2O_2

- place slides labeled with H_2O_2 for 5 minutes in H_2O_2 solution (100 µM)
- remove H_2O_2 solution by dipping slides 3 times in PBS
- place slides in the lysis solution

Treatment with Lysis

- place all slides in the lysis solution
- incubate all slides for at least 1 h at 4°C (max. 24 h)

Prepare:

- 8 metal plates, 8 silicon gaskets, 8 hard plastic covers, 16 iron bolts, 8 silicon covers
- pipette (5-50 µl)
- a large box with ice
- place a moist box with water in the incubator (37°C)

Enzyme treatment

- take FPG and buffer slides out of lysis solution (lysis and H_2O_2 slides remain in the lysis solution)
- wash slides 3 times for 5 min. with buffer F (at the cooling laboratory, 4°C)

- during the waiting period: take FPG stock solution out of the freezer, mix it with 1485 µl of buffer F; vortex briefly and place it on ice immediately
- after the third washing step: dip off puffer F on a paper towel
- place the slides on the metal plates add the 8 silicon gaskets, the hard plastic covers and fix it with iron bolts (work very carefully)
  place it on ice
- FPG slides: add 30 µl of FPG solution to each of the 12 gels
- buffer slides: add 30 µl of buffer F to each of the 12 gels
- add the silicon cover
- place the plates in the moist box on a dry plastic platform, put on the lid
  incubate for 30 min at 37°C

Prepare

at the cooling laboratory :

- electrophoresis
- power supplier
- blank slides
- PBS

Alkaline treatment and Electrophoresis

- get FPG and and buffer slides out of the plates
- place all slides side by side in the electrophoresis tank
- fill gabs with blank slides
- add 2 liters of cold (4°C) electrophoresis buffer
- close the lid of the electrophoreses
- incubate for 20 min (unwinding phase)
- connect to the power cable
- turn the electricity machine on
    - Power Supply needs to be on
    - choose manual (↑↓) and press SET

- voltage: 25 V, press SET
- electricity: on maximal, press SET
- power: on maximal, press SET
- time unit: h, press SET
- time: 30 min, press SET
- after the unwinding phase: press RUN

- electrophoresis runs for 30 min
- wait 1 min, note the ampere (should be around 300 mA)
  if it is too high take out some buffer

Washing steps (4 consecutive)

- put on 2 pairs of gloves
- take slides out of the electrophoresis
- place all slides for 5 minutes in a cuvette with PBS (4°C)
- place all slides for 5 minutes in a cuvette with double distilled water (room temperature)
- place all slides for 15 minutes in a cuvette with 70% ethanol (room temperature)
- place all slides for 15 minutes in a cuvette with pure ethanol (room temperature)
- pour off the ethanol, dab off the ethanol on a paper towel
  place the slides on paper towel in a drawer and dry in the dark over night
- store dried slides in a box at 4°C

Staining and Quantification

- turn the fluorescence microscope on
- turn the computer on
- start Comet 5.5

- dye 2 times 6 gels
- about 5 µl of GelRed (3:10000) were placed on the first 6 gels of the slides
- cover these 6 gels with a coverslip
- count cells in the dark immediately
- count 50 cells per gel (100 cells per subject) (focus gels always first)

Curriculum Vitae

Lebenslauf

Persönliche Daten

Name:	Bianca Guggenberger, Bakk.rer.nat
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Schulische Ausbildung/Studium

Seit Okt. 2012	Master-Studium Ernährungswissenschaften für Lebensmittelqualität und -sicherheit (Hauptuniversität Wien)
Okt.2008 – Sept. 2012	Bachelor-Studium Ernährungswissenschaften (Hauptuniversität Wien) Bachelorarbeit: Traceability of GMO'S
2004 – 2008	Bundes Oberstufen Realgymnasium Hermagor (Kärnten)

Berufliche Erfahrungen

April-Mai 2015	**Stundentische Hilfskraft**: Labortätigkeiten für die Forschungsgruppe von Univ.Prof.Dr. Karl-Heinz, Hauptuniversität Wien Wagner (Teilzeit):
Oktober 2014-Juli 2015	**Tutorin:** Department Ernährungswissenschaften, Hauptuniversität Wien Laborübungen (geringfügige Beschäftigung)

April 2014-Jänner 2015	**Masterarbeit**: Labortätigkeiten (Blutaufbereitung, Durchführung von Comet Assays und Mikroskopieren) Forschungsgruppe von Univ.Prof.Dr. Karl-Heinz Wagner, Hauptuniversität Wien (Vollzeit)
September 2013	**Praktikum:** Ottakringer Brauerei, Wien Labor für Qualitätssicherung Tägliche Durchführung von Analysen, mikrobiologisches Arbeiten, Durchführen von Verkostungen, Produktionskontrolle etc.

Berufliche Erfahrungen in anderen Bereichen

Seit November 2013	**Geringfügige Beschäftigung**: MAG Personal GmbH Servicekraft; Dienste für DO&CO, Palais Coburg, Grand Hotel, The Ring etc.
Juli 2013	**Freiwilligenarbeit ICYE Kenya**: Auslandsaufenthalt in Nairobi, Kenia, Waisenheim Flomina im Soweto Slum
Juli-August 2008	**Auslandsaufenthalt:** Ferialjob in Sarteano, Toskana Servicekraft am Campingplatz „Parco delle Piscine“

Persönliche Fähigkeiten und Kompetenzen

Sprachkenntnisse:	**Muttersprache:** Deutsch **Fremdsprachen:** Englisch (verhandlungssicher) Italienisch (gut)
EDV-Kenntnisse:	Fundierte Kenntnisse in Word, Excel, PowerPoint, SPSS
Sonstige Angaben:	Führerschein B
Hobbies:	Reisen Sport: Laufen, Wander, Krafttraining