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List of Abbreviations

AGE	Advanced glycation end-products
ALA	Alpha-linolenic acid
AO	Antioxidant
BMI	Body mass index
BSA	Bovine serum albumin
CAT	Catalase
CVD	Cardiovascular disease
DM	Diabetes mellitus
DMP	Disease Management Program
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
Endo III	Endonuclease III
FFA	Free fatty acid
FFQ	Food frequency questionnaire
FPG	Formamidopyrimidine glycosylase
GPx	Glutathione peroxidase
H_2O	Water
H_2O_2	Hydrogen peroxide
HbA1c	Hemoglobin A1c
HCl	Hydrochloric acid (concentrated)
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IDDM	Insulin dependent diabetes mellitus
IDDM-T2	Insulin dependent diabetes mellitus type 2
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
KCl	Potassium chloride
KOH	Potassium hydroxide
LMA	Low melting agarose
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide

NaOH	Sodium hydroxide
NGT	Normal glucose tolerance
NIDDM	Non insulin dependent diabetes mellitus
NMA	Normal melting agarose
$O_2^{\cdot -}$	Superoxide anion
ÖDV	Austrian Diabetes Association
$OH^{\cdot}$	Hydroxyl radical
PBS	Phosphate-buffered saline
PUFA	Polyunsaturated fatty acids
QLQ	Quality of life questionnaire
ROS	Reactive oxygen species
RPM	Rounds per minute
SCE	Sister chromatid exchange
SD	Standard derivation
SFA	Saturated fatty acids
SOD	Superoxide dismutase
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
Tris	Trizma Base

1 Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by high blood glucose levels. In the year 2010 worldwide approximately 285 million people were suffering from diabetes according to the International Diabetes Federation. The Austrian diabetes association reports on 600.000 people with diabetes in Austria. The most common form is type 2 diabetes mellitus (T2DM) and accounts for 90–95% of all diabetes cases. Increasing prevalence and incidence of diabetes and its late complications are a great economic burden for the health care system.

Patients with diabetes show greater levels of reactive oxygen species (ROS) and a reduced antioxidant defense system, leading to oxidative stress. It has been suggested that oxidative stress plays a key role in the pathogenesis of late diabetic complications. Oxidative stress, which has also been linked to insulin resistance and beta cell dysfunction, causes increased damage to deoxyribonucleic acid (DNA). There is strong evidence that vegetables and fruits rich in antioxidants and vitamins in combination with plant oils may have protective effects on the amount of oxidative DNA damage.

The aim of the study DIAPLANT was to investigate the positive effects of a dietary intervention with 300 g vegetables and 25 ml plant oil per day for 8 weeks on the risk factors of late diabetic complications in T2DM subjects.

The present thesis is a part of this study and assesses the influence of a dietary intervention on oxidative DNA damage in subjects with insulin-dependent T2DM (IDDM-T2). The levels of oxidative DNA damage were measured by comet assay ex vivo in lymphocytes.

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2 Literature survey

2.1 Diabetes mellitus

2.1.1 Definition and Classification

Diabetes mellitus comprises a group of metabolic disorders and is characterized by high blood sugar levels, which are a consequence of impaired insulin secretion and insulin function that lead to hyperglycemia. Hyperglycemia is correlated with long-term damage, dysfunction and failure of different organs, especially eyes, kidneys, nerves, heart and blood vessels. Specific pathogenic processes are the consequence in diabetes. That comprehends from autoimmune destruction of the ß-cells in the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin. In case there is not enough insulin, or it is not working properly, the blood glucose rises and leads to abnormalities in carbohydrate-, fat-, and protein metabolism [ADA, 2010a]. An etiologic classification of diabetes is shown in table 1.

The status of pre diabetes occurs when abnormalities of glucose metabolism exist but the diagnostic criteria (Tab. 2) for diabetes are not met. These conditions of pre diabetes can be unchanged for many years. If the blood glucose level is abnormal, impaired fasting glucose (IFG) is diagnosed. Determination following the oral glucose tolerance test (measure of two hours after 75 g oral glucose load) is defined as impaired glucose tolerance (IGT). It occurs when beta cell function is inadequately low for a specific degree of insulin sensitivity [SMUSHKIN and VELLA, 2010].

Tab. 1: Etiologic classification of diabetes mellitus according to ADA, 2010

I.	***Type 1 diabetes**: ß-cell destruction, usually leading to absolute insulin deficiency*
	a. Immune mediated
	b. Idiopathic
II.	***Type 2 diabetes**: ranging from predominantly insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with insulin resistance*
III.	***Other specific types of diabetes***
	a. Genetic defects of ß-cell function
	b. Genetic defects in insulin action
	c. Diseases of the exocrine pancreas
	d. Endocrinopathies
	e. Drug or chemical induced
	f. Infections
	g. Uncommon forms of immune-mediated diabetes
	h. Other genetic syndromes sometimes associated with diabetes
IV.	*Gestational diabetes mellitus*

Tab. 2: Diabetes diagnostic criteria, according to ADA, 2010

Diabetes	
Fasting plasma glucose	≥ 7.0 mmol/l (126 mg/dl)
	or
2–h post glucose load	≥ 11.1 mmol/l (200 mg/dl)
Hemoglobin A1c (HbA1c)	≥ 6.5%
IFG and/or IGT (is referred to as having pre diabetes)	
Fasting plasma glucose	5.6 mmol/l to 6.9 mmol/l (100 mg/dl to 125 mg/dl) [IFG]
	and/or
2-h post glucose load	7.8 mmol/l to 11.0 mmol/l (140 mg/dl to 199 mg/dl) [IGT]
	and
HbA1c	5.7 – 6.4%

2.1.2 **Diabetes Mellitus Type 2 (T2DM)**

T2DM accounts for approximately 90-95% of all diabetes cases worldwide. It is formally known as non-insulin-dependent diabetes mellitus (NIDDM), or non-immune-mediated diabetes. This form of diabetes results from an interaction between genetic and environmental factors and appears usually in people over the age of forty. T2DM occurs in individuals who have insulin resistance and relative insulin deficiency. It is often associated with obesity, which itself can induce some degree of insulin resistance and lead to elevated blood glucose levels. Nowadays T2DM becomes more and more common in children, adolescents and young people of all ethnicities and it is associated with a higher genetic predisposition than diabetes mellitus type 1 (T1DM), which is known as insulin-dependent diabetes mellitus (IDDM) [ADA, 2010a].

2.1.2.1 **Pathogenesis**

In a healthy human organism the blood glucose level is kept in a tightly controlled range < 5.6 mmol/l (100 mg/dl) [ADA, 2010a].

The normal glucose tolerance is mainly regulated by the interplay of insulin action and insulin secretion. It is needed to stimulate glucose uptake from the blood various tissues. Depending on the glucose concentration in the blood, insulin is secreted by the beta cells of the Langerhans` islets in the pancreas. Increased insulin levels inhibit glucagon

release from alpha cells in the pancreas. It promotes the conversion of glucose into glycogen and inhibits lipolysis in adipose tissue.

Multiple mechanisms lead to T2DM. Beside the genetic predisposition and environmental aspects also other factors, such as impaired glucose homeostasis, are discussed. Defects in glucose homeostasis decrease insulin resistance and have negative impact on beta cell function. Insulin resistance appears early in this disease, usually when glucose values are still within the normal glucose tolerance range. As glucose tolerance moves from regular to minimally impaired, the insulin secretion that occurs within the first thirty minutes after meal consumption becomes weak. The dysfunction in insulin secretion arises long before the onset of T2DM. It is not decrease of insulin resistance that causes blood glucose values to induce IGT, but it is the dysfunction of beta cells [LEAHY, 2005].

In addition to increased insulin resistance a steady decline of beta cells follows, the body cannot transfer glucose to the cells any longer and results in diabetes. Beta cell dysfunction is a critical stage in the pathogenesis of T2DM [STUMVOLL et al., 2005].

High triglycerides and free fatty acids, which are typical for T2DM also contribute to the pathogenesis of the disease. Experimental studies discussed a causative event in the transition from normal to abnormal glucose tolerance and propose an interaction of glucose toxicity and lipotoxicity, termed glucolipotoxicity [LEAHY, 2005].

Glucose toxicity leads to an irreversible damage of beta cells caused by chronic exposure to hyperglycemia. Robertson et al. hypothesised in their study that decreased insulin action and secretion are caused by reduced insulin gene expression. Also chronic oxidative stress may be an important mechanism for glucose toxicity [ROBERTSON et al., 2003].

T2DM is treated with healthy diet, physical activity and a combination of oral hyperglycaemic drug application with lipid-lowering, antihypertensive and antiplatelet therapy. If stable metabolic control is not achievable exogenous insulin is required [ADA, 2010a].

2.1.2.2 **Complications**

Diabetes is one of the leading causes of death and affects many major organ systems where the most significant, and often fatal, diabetes complications occur. The mechanisms involve the direct toxic effects of high glucose levels, along with the

impact of elevated blood pressure, abnormal lipid levels and both functional and structural abnormalities of blood vessels. In addition to acute complications like severely elevated blood sugar levels and abnormally low blood sugar levels, chronic complications develop gradually. Long-term complications are related to blood vessel diseases and are generally classified into small vessel disease and large vessel disease [IDF,2010a].

The management of T2DM should be further optimized to reduce the incidence of complications. Type 2 diabetics have in comparison to healthy individuals shorter life expectancy and reduced quality of life due to late diabetes complications [RAKOVAC et al., 2009].

2.1.2.2.1 **Microvascular complications**

Microvascular complications involve the eyes, kidneys and nerves. Diabetes can harm vision and initiate diabetic retinopathy. Both macular edema, caused by a fluid accumulation behind the retina of the eye and impair small blood vessels in the back of the eye, in consequence of the leakage of protein and blood in the retina, induce blindness. In addition to blindness, diabetes also increases the risk of cataracts and glaucoma [ADA, 2010b].

Diabetic nephropathy includes kidney damage. Impaired small blood vessels in the kidneys cause the leakage of protein into urine. The accumulation of toxic waste products in the blood leads to dialysis or organ transplantation.

When blood glucose and blood pressure are not controlled enough diabetes can impair nerves. The overload of sugar can injure the walls of the capillaries that nourish the nerves, especially in the legs. Nerve damage in these areas is called peripheral neuropathy or diabetic neuropathy. Tingling, numbness, burning or pain lead to a loss of sense of feeling in the affected limbs. Minor foot injuries can produce serious infections, ulcers, gangrene and may require amputation of infected parts. Nerves of the digestive tract can also be affected and initiate nausea, vomiting, diarrhea or constipation and erectile dysfunction. Beside these major microvascular diseases diabetes may lead to a higher susceptibility to skin and oral mucosa problems. Itching, bacterial-, fungal- and gum infections may occur which might also lead to hearing impairment in cause of diabetes mellitus [IDF, 2010a].

2.1.2.2.2 **Macrovascular complications**

Diabetes leads to accelerate hardening of arteries of the larger blood vessels and initiates dramatically an increase of risk in coronary heart diseases. Cardiovascular disease (CVD) is the major cause of death in diabetes [ADA, 2010b]. According to the "American Heart Association" about 65 % of diabetics die of some type of heart or blood vessel disease [AHA, 2011].

To reduce macrovascular complications it is of primary importance to control hypertension, hyperlipidemia, insulin resistance and obesity [JÖNSSON, 2002]. In addition diabetes may lead to lower bone mineral density and increase the risk of osteoporosis. The poorer blood sugar levels are controlled, the greater the risk of Alzheimer´s disease and vascular dementia appears to be. Cardiovascular problems caused by diabetes might lead to dementia by blocking blood flow to the brain or cause stroke. Too much insulin in the blood leads to brain damaging inflammations, while lack of insulin in the brain deprives brain cells of glucose [ADA, 2010b].

2.1.3 **Prevalence of late diabetic complications in Austria**

The prevalence of long-term complications in Austria is high but comparable with other European countries. In the Austrian study "Health status of type 2 diabetic patients - perspective of a quality improvement initiative" data were collected between 1997 and 2007 to estimate the incidence of late complications of type 2 diabetic patients. Data from 23 641 subjects were analysed. Each fifth Austrian diabetic patient was affected at least by one kind of complication like amputation, heart attack, stroke, blindness or dialysis [RAKOVAC et al., 2009].

2.1.4 **Epidemiology**

2.1.4.1 **Worldwide**

Diabetes is one of the most prevalent non-communicable diseases globally. In accordance with the "Diabetes Atlas" of the International Diabetes Federation (IDF) it is the fourth or fifth leading cause of death in most high-income countries [IDF, 2010b].

Studies from 91 countries were used to describe the world prevalence of diabetes. In the year 2010 they estimated 285 million people living with diabetes. The study-population contains adults aged between 20-79 years. The majority of diabetics in industrialized countries are aged over 60 years. Affected people are younger in developing countries.

The uppermost regional prevalence was counted for North America, followed by the Eastern Mediterranean, Middle-East and South Asia [SHAW et al., 2010].
The incidence of T2DM is increasing worldwide, often in countries that cannot achieve the resulting medical and financial burdens [LEAHY, 2005].
The prevalence will increase to 7.7% or 439 million diabetic people in the year 2030. The African region is expected to have the largest proportional growing in adult diabetes and North America will continue to have the world´s highest prevalence. Over the next 20 years population growth, ageing of residence group and urbanization associated with lifestyle change is predicted to lead to a 54% rise of the total number of diabetics [SHAW et al., 2010].

2.1.4.2 **Austria**

About 600 000 Austrians suffered from diabetes mellitus in the year 2010 and only 420 000 people are official diagnosed with this disease. The estimated number of unreported cases is about 30%, similar to other developed countries. It is recognized that in Austria 20% of all people at risk already have clinical late complications of diabetes by their first diagnose of T2DM. These complications could be prevented if the diagnosis occurs in time. The process of an early diagnosis, the right treatment and the prevention concept has to be better supported by the Austrian health system. Especially in societies with major changes in the type of diet consumed, reductions in physical activity, and increases in overweight and obesity diabetes develops strongly. 9% of people with overweight and more than 15% with obesity develop diabetes, whereas only 3% of all people with normal weight suffer from diabetes. Educational advertising and sensitization become more important in new instructions like the “Disease Management Program” (DMP). An agenda called “Therapy active” for T2DM may affect positively the reduction of late chronic complications and with it the total cost caused by diabetes [ÖDG, 2010].

2.1.5 **Economic Aspects of Diabetes**

Increasing prevalence of diabetes and high incidence of late complications lead to significant problems for the health care system in developed and non-developed countries. The management of diabetics should be further optimized in order to reduce the prevalence of long-term complications. Comprehensive economic data on the costs

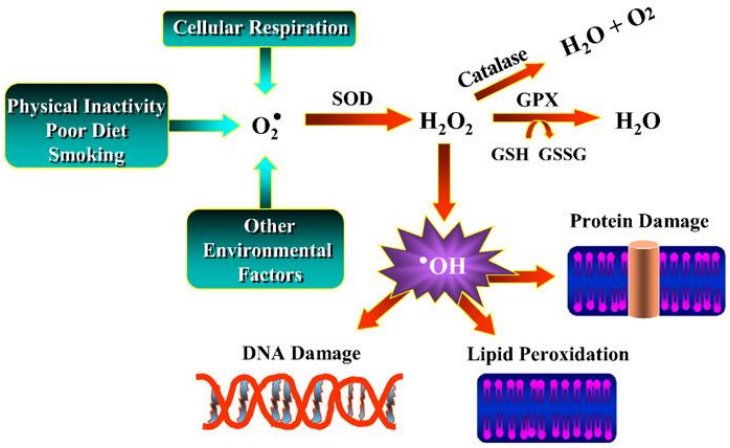
of diabetes are required for policy decisions, in order to upgrade the allocation of resources and to evaluate the success of different attempts for disease management. The "CODE-2 study" described the first coordinated effort to measure the cost caused by people with T2DM in Europe. This study calculated total healthcare costs for more than 7000 patients in eight countries (Belgium, France, Germany, Italy, Netherlands, Spain, Sweden and United Kingdom). The total costs of T2DM in these countries were estimated at EUR 29 billion. The average annual costs per patient were estimated at EUR 2834,-.The greatest direct costs are due to CVD. Most of recruited patients in the study were older than 65 years and were treated with oral anti diabetic agents [JÖNSSON, 2002].

2.2 Reactive oxygen species and oxidative stress

2.2.1 Reactive oxygen species

Free radicals, such as reactive oxygen species (ROS), are produced in biological systems. A free radical is any molecule that holds in an atomic or molecular orbit one or more unpaired electrons, which makes them highly reactive. Free radicals can be formed by losing or gaining a single electron or when a covalent bond is broken [HALLIWELL and GUTTERIDGE, 2007].

ROS are highly reactive and derivatives of the oxygen metabolism. The most commonly known ROS in the organism are the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). The superoxide anion is called the primary radical. The main source for the formation of $O_2^{\cdot -}$ is the mitochondrial respiratory chain. But also exogenous sources like smoking, unhealthy diet and physical inactivity are responsible for the elevated production of $O_2^{\cdot -}$ in biological systems. Superoxide dismutase (SOD) converts mitochondrial superoxide to H_2O_2 and produces $OH^{\bullet}$ when not detoxified by the enzymatic defence system. If the antioxidant system is not able to inactivate ROS, they can damage DNA, proteins and lipids [ROBERTS and SINDHU, 2009] (Fig. 1).



GPx = glutathione peroxidase, GSH = reduced glutathione, GSSG = oxidised glutathione

Fig. 1: Generation and detoxification of ROS

(Roberts and Sindhu, 2009)

2.2.2 **Oxidative stress**

Oxidative stress is defined as an imbalance between pro-oxidants and antioxidants. It can lead to molecular damages. Oxidative stress results from an increased level of ROS and/or decreased antioxidant defense (Fig. 2). High levels of oxidative stress lead to DNA damage and cell death and often the apoptotic cascade is initiated. Double-strand breaks are often linked to apoptosis [HALLIWELL and GUTTERIDGE, 2007].

Diabetes mellitus is associated with elevated production of free radicals and reduced plasma antioxidant status. These events lead to increased oxidative stress [RAHIMI et al., 2005].

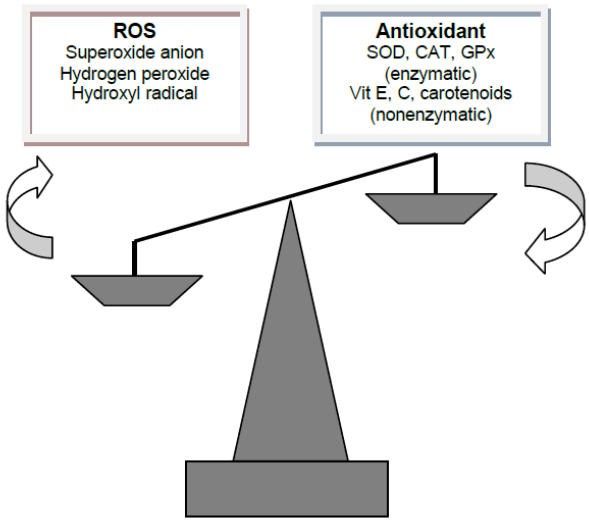


Fig. 2: Imbalance between pro-oxidants and antioxidants

(modified after Elmadfa and Leitzmann, 2004)

2.2.3 Oxidative stress and T2DM

2.2.3.1 Sources of oxidative stress

Oxidative stress plays a key role in the pathogenesis of late diabetic complications and has been linked to insulin resistance as well as to beta cell dysfunction. Diabetics show increased levels of oxidative stress leading to cell damages induced by free radicals and lower plasma antioxidant status. Hyperglycemia in diabetic patients is the main source for oxidative stress. It can induce oxidative stress via several pathways, including the autoxidation of glucose, generation of advanced glycation end-products (AGE) and the polyol pathway activation [JAY et al., 2006].

Free fatty acids (FFA) and leptin are other sources of elevated oxidative stress in diabetes. Excessive levels of FFA lead to an overproduction of nicotinamide adenine dinucleotide (NADH), which may contribute to an increased formation of $O_2^{\cdot -}$ [JAY et al., 2006] (Fig. 3).

2.2.3.1.1 Oxidative stress and beta cell dysfunction

Elevated glucose and lipid concentrations induce beta cell dysfunction. Insufficiency of beta cells leads to excessive production of ROS. The effect of oxidative stress in beta cells is strengthened because they have a low level of antioxidant enzymes. As a consequence, oxidative damage in beta cells is enhanced and lead to increased apoptosis. Dysfunction of beta cells with reduced insulin secretion is evident before the progression of hyperglycemia [NEWSHOLME et al., 2009].

2.2.3.1.2 Oxidative stress and insulin resistance

Oxidative stress is also characterized by insulin resistance in several tissues such as liver, fat and muscle. There is strong evidence that oxidative stress is related to obesity and T2DM [WEI et al., 2008]. Data from the Framingham Offspring Study showed a positive association between oxidative stress and insulin resistance among 528 obese subjects [MEIGS et al., 2007].

It has been shown in animals models in vitro, that the intake of glucose in muscle cells is impaired in adipocytes and L6-myocytes after exposure to oxidative stress. These data indicate that oxidative stress is involved in the development of insulin resistance [MADDUX et al., 2001; RUDICH et al., 1999].

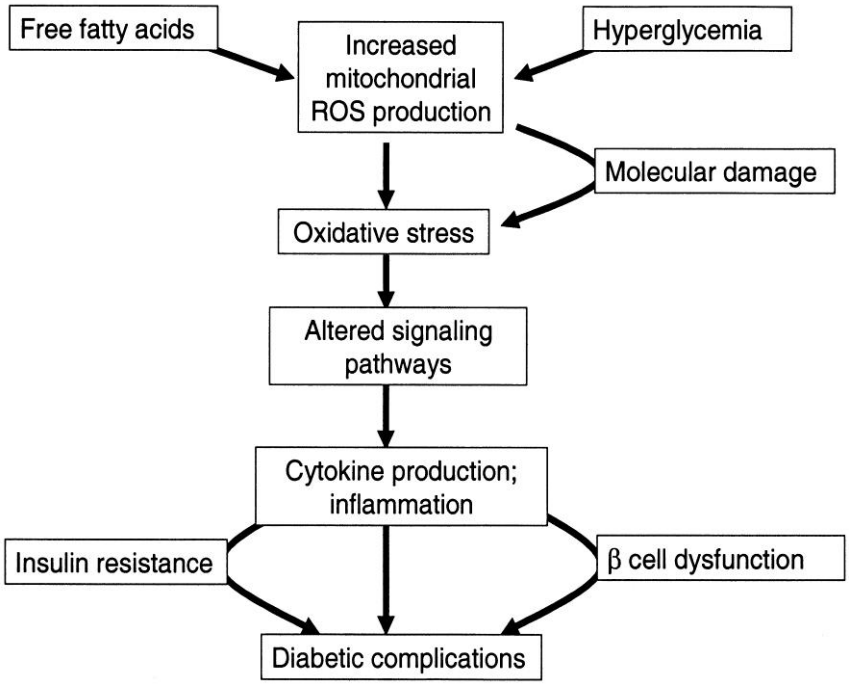


Fig. 3: Overview of the development of diabetic complications

(Evans et al., 2002)

2.2.3.2 **Antioxidant defences against oxidative stress**

An antioxidant is any substance that prevents, removes or delay oxidative damage to DNA, lipids, proteins and other molecules. Antioxidants are synthesized endogenously or must be obtained from the diet [HALLIWELL and GUTHERIDGE, 2007].

2.2.3.2.1 **Enzymatic antioxidants**

Several enzymes can detoxify reactive molecules, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). In subjects with diabetes the activity of these enzymes can be decreased, leading to disabled responses of defense and increased oxidative stress [MOKINI et al., 2010].

Superoxide dismutase is present as CuZnSOD in the cytosol and as MnSOD in the mitochondria. It can dismutate $O_2^{\cdot -}$ immediately to H_2O_2. Together with catalase in the lysosomes and glutathione peroxidase in the mitochondria H_2O_2 is detoxified to H_2O and O_2 [JOHANSEN et al., 2005] (Tab. 3).

Tab. 3: Enzymatic antioxidants and their mechanism
(modified after Lee et al.,2004)

Superoxide dismutase	$2\ O_2^{\cdot -} + 2\ H^+ \rightarrow H_2O_2 + O_2$
Catalase	$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$
Glutathione peroxidase	$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$

GSH: reduced glutathione, GSSG: oxidised glutathione

2.2.3.2.2 **Nonenzymatic antioxidants**

The most common nonenzymatic antioxidants include Vitamin E (family of different tocopherols and tocotrienols) and C, carotenoids and polyphenols. The intake of appropriate amounts of vegetables and fruits rich in natural antioxidants is able to reduce oxidative stress [LEE et al., 2004].

Vitamin E is the major fat-soluble antioxidant that prevents lipid peroxidation. The most active form in humans is α-tocopherol. It is a scavenger of ROS such as $O_2^{\cdot -}$ and $^{\cdot}OH$ and can be regenerated by Vitamin C (ascorbic acid) .Vitamin C is water soluble. It scavenges $O_2^{\cdot -}$ and $^{\cdot}OH$ and is a quencher of singlet O_2. Ascorbic acid regenerates α-tocopherol from α-tocopherol radical [LEE et al., 2004].

Carotenoids are fat-soluble and have the ability to scavenge ROS. The most effective quencher of singlet O_2 are β-carotene and lycopene. Polyphenols are radical scavengers and can inhibit the peroxidation of lipids. Several can also react with $O_2^{\cdot -}$. Flavonoids such as catechin, quercetin and caffeic acid are very potent antioxidants [HALLIWELL and GUTHERIDGE, 2007].

Antioxidants and nutrients increase the resistance of cells against oxidative stress. The treatment of lymphocytes ex vivo with an oxidant (mainly with H_2O_2) gives information about the antioxidant capacity [DUSINSKA and COLLINS, 2008].

2.3 **Comet Assay**

The comet assay is a rapid and very sensitive technique widely used for the quantification of DNA damage in mammalian cells. The assay was designed by Ostling and Johanson in 1984 [OSTLING and JOHANSON, 1984] for the detection of double strand breaks. Due to further developments by Singh et al. [SINGH et al., 1988] and Olive et al. [OLIVE et al., 1991] different forms of DNA damage can be measured today. Under alkaline conditions the comet assay allows the analysis of single- and

double-strand breaks and alkaline labile sites. More recently, oxidized bases in the DNA can also be detected with specific enzymes Endonuclease III (Endo III) and Formamidopyrimidine glycosylase (FPG). The enzymes convert oxidised bases into additional DNA strand breaks which can be detected. Comet assay has been used in a number of dietary intervention studies, in clinical and biomonitoring studies [COLLINS, 2004; KASSIE et al., 2000; MØLLER et al., 2000].

2.3.1 **Oxidative DNA damage in DM measured by comet assay**

In a review by Møller and Loft, it is shown that the levels of DNA damage are elevated in patients with diabetes [MØLLER and LOFT, 2002]. Dusinska and Collins reported higher levels of oxidised bases in the DNA of lymphocytes from diabetic patients [DUSINSKA and COLLINS, 2008]. Particularly glycaemic control in diabetics is important for the significance of DNA events. It was seen that subjects with poor glycaemic control have elevated levels of DNA damage in comparison to good controlled diabetics and healthy subjects [HANNON-FLETCHER et al., 2000].

According to the literature, conflicting results were reported in subjects with diabetes and DNA damage.

Collins et al. reported, that DNA strand breaks and Endo III-sensitive sites in lymphocytes were significantly higher in T1DM (IDDM) subjects with poor glycaemic control when compared to controls. FPG-sensitive sites were not significantly increased in the diabetes group [COLLINS et al., 1998].

On the other hand, Anderson et al. and Hannon-Fletcher et al. found no significant differences in the levels of DNA damage from diabetic subjects (IDDM and NIDDM) with good glycaemic control when compared to the healthy controls [ANDERSON et al., 1998; HANNON-FLETCHER et al., 2000].

Several studies reported, that in leukocytes increased levels of FPG-sensitive sites in subjects with T2DM (NIDDM) when compared with healthy individuals were seen [DINÇER et al.; 2002; PITOZZI et al., 2003].

Another study including 52 patients with T2DM (NIDDM and IDDM-T2) reported also significant higher amounts of Endo III- and FPG-sensitive oxidative DNA damage in lymphocytes when compared to the healthy control group (n = 55) [BLASIAK et al., 2004].

Choi et al. found a correlation between HbA1c levels in subjects with T2DM (NIDDM) and damages of DNA in lymphocytes. The results of this cross-sectional study (n = 427) showed that oxidative DNA damage is significantly increased in diabetics with poor glycaemic control and is associated with a lower status of plasma ascorbic acid [CHOI et al., 2005].

Song et al. studied the amount of DNA damage in 92 subjects with normal glucose tolerance (NGT), 78 subjects with impaired glucose regulation (IGR), 113 patients with T2DM (NIDDM), which were newly diagnosed. The newly diagnosed individuals showed significant higher FPG-sensitive sites compared to the IGR subjects. The subjects with diagnosed diabetes had an elevated level of HbA1c (8.01 ± 1.89). In patients with IGR higher levels of DNA damage was observed than in subjects with NGT [SONG et al., 2007].

Another study investigated the differences of DNA damage in leukocytes from 25 T2DM (NIDDM) subjects undergoing hemodialysis in comparison to 20 healthy controls. When compared to the controls, the patients with T2DM showed elevated levels of DNA damage [BRAMBILLA BAGATINI et al., 2008].

A study with 39 T2DM (NIDDM) subjects (41 - 79 years) and 18 healthy controls aged between (35 - 70 years) reported on the oxidative DNA damage in leukocytes. The results support the former findings that oxidative DNA damage is elevated in subjects with diabetes and that poorly controlled subjects have significantly decreased levels in plasma antioxidant capacity [LODOVICI et al., 2008].

In a more recent case-control study with 71 T2DM (NIDDM) subjects (40 - 70 years) and 14 healthy individuals (40 - 50 years), no significant differences in the levels of DNA damage were found between healthy controls and long term diabetics [IBARRA-COSTILLA et al., 2010].

2.3.2 **Intervention studies and Comet Assay**

2.3.2.1 **The effect of antioxidants (AOs) on oxidative DNA damage**

The assumption that nutritional modulations can protect against oxidative DNA damage prompt the investigation whether it is possible to modify the level of DNA damage with antioxidative supplements and they increase the resistance of lymphocyte DNA to oxidation. Antioxidant (AO) intervention studies include diets with one or more food

items as well as single or multiple AO treatment to increase the levels of AOs or specific types of vitamins and minerals. Several trials investigated the potential of AOs.

2.3.2.1.1 **Antioxidant Supplementation trials**

In a study by Duthie et al. 100 healthy, smoking and nonsmoking males aged 50 – 59 years were randomly assigned to the supplement or placebo group. The supplementation lasted for 20 weeks and contained a daily intake of 100 mg vitamin C, 280 mg α-tocopherol and 25 mg ß-carotene. The results of endogenous DNA damage showed that smokers had significantly higher levels of oxidised pyrimidines than nonsmokers. After 20 weeks of supplementation a highly significant decrease in oxidative base damage could be shown in both smokers and nonsmokers. Furthermore lymphocytes from subjects receiving antioxidative supplements were more resistant to H_2O_2 (0.1 mM or 0.3 mM) [DUTHIE et al., 1996].

Another study performed by Lee et al. with 15 healthy male smokers and 5 healthy male non-smokers aged between 19 - 31 years investigated the effects of different antioxidants on DNA damage. Smokers received 200 IU of vitamin E, 500 mg of Vitamin C, 9 mg of β-carotene or 1.8 g of red ginseng or a placebo daily for a period of 4 weeks. The results showed a significant decrease in oxidative DNA damage in subjects supplemented with Vitamin E and red ginseng after 4 weeks in comparison to baseline. Smokers with Vitamin C and β-carotene supplementation had slightly reduced DNA damage without being significant [LEE et al., 1998].

Zhao et al. investigated the effect of carotenoid supplementation on 37 elderly healthy women (50 - 70 years) against oxidative DNA damage. The subjects received either mixed carotenoids with lutein, β-carotene and lycopene (4 mg each), or 12 mg of a single carotenoid (lutein, β-carotene or lycopene) or placebo for 56 days. Blood samplings were taken every 2 weeks. After 15 days of supplementation, the groups with mixed carotenoids and with β-carotene showed significantly lower DNA damage. At the end of the intervention period, all supplemented groups had significantly lower DNA damage [ZHAO et al., 2006].

The impact of carotenoids on DNA damage was also analysed by Devaraj et al. In this study 77 healthy subjects aged ≥ 40 years consumed for 8 weeks 6.5, 15 or 30 mg lycopene per day or placebo. All subjects were asked to maintain a lycopene-restricted diet 2 weeks before starting and during the intervention period. Subjects, receiving 30

mg of lycopene, had significantly lower level of DNA damage after 8 weeks of supplementation [DEVARAJ et al., 2008].

2.3.2.1.2 **Food intervention and Food extract trials**

A study recruited 23 healthy, non-smoking males aged 27 – 40 years. The volunteers were asked to consume vegetable products with lunch consecutively for a 2 week period each. The daily intake was 330 ml carrot-, 330 ml tomato juice and 10 g dried spinach powder dissolved in water or milk. The levels of DNA damage were significantly higher at the beginning of the experiment (before intervention) than during the intervention period with vegetable products. The treatment with carrot juice caused a significant reduction in oxidised pyrimidine bases of the DNA. The intervention with tomato juice or spinach did not cause any reduction in oxidised pyrimidines. Pool-Zobel et al. also suggest that carotenoids containing plant products can protect against oxidative DNA damage but the beneficial effects vary with the type of products [POOL-ZOBEL et al., 1997].

The effect of a daily supplementation with extracts of vegetables and fruits to reduce the amount of DNA damage in peripheral lymphocytes was investigated in a study with 20 elderly subjects (mean age 68 years). The volunteers were treated with 850 mg fruit powder and 750 mg vegetable powder per day for a period of 80 days. Data demonstrated a significant decrease of DNA damage following supplementation. There was link to sex, age or smoking status [SMITH et al., 1999].

In a crossover designed study 10 healthy non-smoking women (23 ± 1.1 years) were divided into two groups. They were given either a diet of 60 g tomato puree (16.5 mg lycopene and 0.6 mg ß-carotene) or a tomato free diet for 21 days each. Subjects consumed the uncooked tomatoes with 10 g olive oil and 70 g pasta. For tomato free diet the subjects received pasta with olive oil. The relative tail moment was significantly decreased after the consumption of the tomato puree, and showed a significant increased resistance of the lymphocytes to oxidative stress [RISO et al., 1999].

In another study by Porrini and Riso 9 young adult women (25.4 ± 2.2 years) were treated with 25 g tomato puree containing 7 mg lycopene and 0.3 mg ß-carotene for 14 days. After a run-in period (1 week) with a diet low in carotenoids they consumed uncooked tomato puree with pasta and 5 g of olive oil for lunch for 14 days. The levels of DNA damage decreased significantly in comparison to baseline after treatment with

H_2O_2 (500 µmol/L). The levels of DNA damage were reduced by 50% [PORRINI and RISO, 2000].

Porrini et al. performed a dietary controlled intervention study with 9 non-smoking females (25.2 ± 2.2 years). They consumed a diet low in carotenoids with one portion of spinach (150 g) per day for three weeks. After a 2 week wash-out period they were asked to consume spinach (150 g) and tomato puree (25 g) together for further 3 weeks, with 10 g olive oil. To measure H_2O_2 resistance the slides were treated with H_2O_2 (500 µmol/L). After both supplementation periods a significant increase in resistance to H_2O_2 could be shown [PORRINI et al., 2002].

In a parallel designed study performed by Møller et al. 43 subjects were randomized into three groups, receiving 600 g of fruits and vegetables, or a supplement with the equal amount of AOs and minerals or a placebo for a period of 24 days. Subjects were between 21 – 56 years old. There was no effect on oxidative DNA damage after consumption of fruit and vegetables or supplements. Lymphocyte resistance to H_2O_2 treatment did not differ between the three groups. These unchanged effects may indicate that the inherent antioxidant defense mechanisms are sufficient to protect circulating mononuclear blood cells from reactive oxygen species [MØLLER et al., 2003].

In a further crossover study by Collins et al. 14 healthy nonsmoking volunteers (8 females, 6 males) (26 – 54 years) participated. The subjects were allocated randomly into three groups and each of which was given a different order of kiwifruit doses. A daily intake of 1, 2 or 3 kiwifruits lasted 3 weeks separated by a 2-week washout period. The amount of DNA damage was not related to the number of fruits consumed. The levels of DNA strand breaks in the lymphocytes after treatment with 100 µM H_2O_2 were significantly lower after the consumption of kiwifruit than in the washout-period and indicate an increased AO capacity. Oxidised bases (pyrimidines and purines) showed significantly decreased levels on oxidised DNA damage [COLLINS et al., 2003].

Astley et al. showed in a study with 64 healthy males (18 – 50 years) that after carotenoid supplementation or intake of foods rich in carotenoids for 3 weeks no effects on oxidative DNA damage and resistance to H_2O_2 were seen [ASTLEY et al., 2004].

Gill et al. performed a parallel design dietary intervention study among 20 healthy individuals (mean age of 25.5 years) with a daily intake of 113 g cruciferous and legume sprouts for 2 weeks. The results showed in the intervention group significantly

higher resistance to H2O2-induced damage to DNA in comparison to the control group [GILL et al., 2004].

26 healthy individuals were recruited for a double-blind, crossover study and were randomly divided in two groups. Group one started with placebo intake, followed by a wash-out period and the treatment with a tomato drink, containing 5.7 mg of lycopene, 3.7 mg of phytoene, 2.7 mg of phytofluene, 1 mg of ß-carotene, and 1.8 mg α-tocopherol (mean age 25.7 ± 2.1 years). Group two started with the tomato drink, followed by a wash-out phase and the placebo intake (mean age of 25.9 ± 3.4 years). Each period lasted 26 days. There were no significant changes in endogenous lymphocyte DNA damage following the treatment with placebo or the tomato drink. Riso et al. hypothesized, that there were no effects on markers of DNA damage because the basal levels of the healthy individuals were very low (2 – 3%) [RISO et al., 2006]

A recent intervention study included 8 healthy non-smoking volunteers in the average age of 33 ± 7 years. They consumed 300 g of Brussels sprouts daily over a period of 6 days. The consumption leaded to a decrease in oxidative DNA-damage. Measurements showed a significant decrease by 45% in oxidised pyrimidine bases and a reduction by 39% in DNA damage after treatment with H_2O_2 [HOELZL et al., 2008].

In a more recent study the participants (n = 8) consumed 225 g per day homogenized spinach over a period of 16 days. The results showed a significant decline of H_2O_2-induced damage and the reduction of oxidised pyrimidines was still significant after spinach consumption [MOSER et al., 2011].

2.3.2.2 **Intervention studies with AO in diabetics and DNA damage**

AOs may have more protective effects on DNA damage among subjects with diabetes, who suffer from higher levels of oxidative stress [MØLLER and LOFT; 2006].

An 8 week intervention study investigated 42 subjects with T1DM (IDDM) and 31 healthy individuals. In the randomized prospective double-blind placebo-controlled trial the subjects were treated with a supplement of 400 IU α-tocopherol or placebo per day. In week 4 and 8 and 4 weeks after the intervention blood samplings were taken. There were neither significant effects on DNA strand breaks nor on H_2O_2 resistance in both groups [ASTLEY et al., 1999].

The effect of a high flavonol diet (supplemented) and a low-flavonol diet was investigated in 10 patients with T2DM (NIDDM). It was a crossover study with an

intervention period of 2 weeks. The subjects received a low-flavonol diet or a low-flavonol diet supplemented with 400 g of onion or onion, tomato ketchup and herb supplement and 6 cups of black tea per day. A significant resistance to damage of H_2O_2 to DNA was seen in the high-flavonol diet (supplemented) in comparison to the low-flavonol diet. The results showed no change on Endo III-sensitive sites [LEAN et al., 1999].

Sampson et al. studied 40 patients with T2DM (NIDDM) and 30 matched controls in a randomized, double–blind and placebo-controlled trial. The subjects received a supplement of 400 IU α-tocopherol or placebo daily. The intervention period lasted 8 weeks followed by a 4 week wash-out phase. Blood was collected at baseline, at week 8 and week 12. The results showed no change in any of the groups [SAMPSON et al., 2001].

In a study, 32 subjects with IDDM (T1DM), NIDDM (T2DM) and healthy controls were supplemented with 900 mg α-tocopherol per day for 12 weeks. In contrast, 28 patients consumed for the same time period a placebo. Blood samplings were taken before and after intervention. All subjects treated with α-tocopherol showed significant lower levels of DNA damage after intervention. The NIDDM patients showed more damage of DNA than the IDDM subjects [ŞARDAŞ et al., 2001].

2.3.2.3 **The effect of fatty acids on oxidative DNA damage**

Plant oils are important sources of polyunsaturated fatty acids (PUFAs) and AOs for the human organism. Especially omega-3 (α-linolenic acid) and omega-6 (linoleic acid) PUFAs that cannot be synthesized by the body represent essential components in the human diet [TURNER et al., 2010].

It is recommended to consume omega-3 and omega-6 fatty acids at a ratio of 1:5 [DACH, 2000].

In traditional diets the proportion of omega-6 fatty acids is much higher. Saturated fatty acids (SFAs) lead to increased LDL-cholesterol levels, which contributes to an increased risk for CVD. It is suggested that PUFAs have beneficial effects on lipid metabolism but on the other hand they might increase susceptibility to lipid peroxidation in LDL [PÉREZ-JIMÉNEZ et al., 2002].

Omega-6 fatty acids increase blood viscosity, vasospasm and vasoconstriction and decrease bleeding time. In addition to the beneficial effect of omega 3 fatty acids which

have antiinflammatory, antithrombotic, antiarrhythmic, hypolipidemic and vasodilatory properties, they show positive effects in the secondary prevention of CVD, hypertension and T2DM. Alpha-linolenic acid (ALA; 18:3 n - 3), the essential precursor of omega-3 fatty acids can be converted to long chain omega-3 PUFAs which are also found in marine oils. ALA can be found in green leafy vegetables, flaxseeds, rapeseeds and walnuts and their oils are metabolized in the human body through elongation and desaturation to eicosapentaenoic acid (EPA; 20:5 n - 3) and docosahexaenoic acid (DHA; 22:6 n-3) [SIMOPOULOS, 2003].

In a cross over study 21 healthy non-smoking males (28.9 ± 1.3 years) were selected into two groups. The first group was treated with a diet containing 5% PUFA as food energy for a period of 4 weeks and after a 10 week wash-out period they consumed a 15% PUFA diet for another 4 weeks. The second group followed an identical protocol except that they started with the 15% PUFA diet. In addition the volunteers were treated with or without α-tocopherol acetate (80 mg per day) and the intake result a level of α-tocopherol in the range of 5 -7 mg/day. DNA damage induced by 200 μM H_2O_2 and oxidised pyrimidines were significantly decreased after the low PUFA diet but significantly increased following the 15% PUFA diet when α-tocopherol levels were in the range of 5 – 7 mg/day. These effects could not be seen in individuals taking 80 mg α-tocopherol per day. This study suggested that an increased amount of PUFA in the diet should only be recommended when an adequate antioxidant intake is ensured [JENKINSON et al., 1999].

Due to the fact that AOs have an important role in preventing the progression of long-term complications in diabetes, a study by Balkis Budin et al. was aimed of investigating the effect of ALA supplementation on plasma lipids, oxidative stress and vascular changes in diabetic and non-diabetic rats. Diabetes was induced after an overnight fast by single intravenous injection of streptozotosin (50 mg/kg body weight). The diabetic rats with blood glucose levels > 15.0 mmol/L represented the diabetic group and were divided in a supplementation group with ALA (n = 8) and non-supplementation group (n = 8). The results showed significantly higher levels of tail moment in diabetic rats compared to the non-diabetic rats. There was a significant increase in the levels of DNA damage in non-supplemented diabetic rats. The ALA supplementation inhibited an increase in DNA damage in diabetic rats. The amount of

DNA damage in the ALA supplemented group was significantly lower when compared to diabetic rats without ALA supplementation, but it was respectively higher when compared to the non-diabetic rats. Balkis Budin et al. concluded that DNA damage that appears in diabetic rats can be repaired by ALA treatment [BALKIS BUDIN et al., 2009].

Another indicator of DNA damage is the measurement of sister chromatid exchange (SCE). In a double-blind, cross-over study 20 normal healthy non-smoking males aged 19 – 31 years participated. The aim was to investigate the effect of plant-oils on DNA damage. The diets contained 30% energy as fat including either 80 g of corn oil (20 mg α-tocopherol, 100 mg γ-tocopherol) or 80 g of a mix of olive- and sunflower oil (24 mg α-tocopherol, 2.4 mg γ-tocopherol). The results indicate that corn oil intake rich in γ-tocopherol in spite of the high amount in omega-6 PUFA (P/S = 4.2) gives better protection against DNA damage than a mix of olive and sunflower oil, rich in α-tocopherol [ELMADFA and PARK, 1999].

In a pilot study the effect of almond consumption in 30 healthy regular smokers was studied. The subjects were randomly divided into three groups. The subjects of group 1 and 2 received 84 g and 168 g of almonds per day respectively for a period of 4 weeks. The third group did not get any almonds. Compared with the control group the results showed decreased levels of single strand DNA breaks in the two groups with almond consumption. Jia et al. concluded that increased almond consumption can prevent DNA damage in smokers [JIA et al., 2006].

In a larger placebo-controlled, cross-over clinical study 60 smokers aged 21.8 ± 0.2 years were randomly assigned into two groups. Their diet was supplemented with 84 g whole almond powder or 120 g pork (to control for calories) for 4 weeks with a washout-period of 4 weeks between treatment periods. In addition a reference group with 30 nonsmoking males aged 21.± 0.4 years was supplemented with 120 g pork per day. The levels of DNA strand breaks in lymphocytes were higher in smokers than in nonsmokers. The daily almond intake reduced DNA strand breaks by 34% in smokers compared with pretreatment value. Pork intake did not have any effect on DNA damage. After the intervention with almonds the magnitude of DNA strand breaks in the smokers was not significantly different from non-smokers. Li et al. hypothesize that

vitamin E, antioxidant phenolic acids, flavonoids and other polyphenols are responsible for the reduction of lymphocyte DNA strand breaks in smokers [LI et al., 2007].

2.3.3 **Conclusion comet assay literature**

These reported intervention studies demonstrate different results on the effects of oxidised DNA damage in lymphocytes. One main reason for these heterogeneous results could be the low basal levels of DNA damage in healthy individuals. In many cases only a small number of volunteers participated in the studies. On the other hand the studies present a variety of intervention strategies, supplement- or dietary interventions, and therefore the period of consumption was often too short to measure significant effects in oxidised DNA damage or other not effective. Particularly there were only a few studies with intervention according to the levels of DNA damage in diabetics detected by comet assay.

Therefore, we investigated in our study the impact of a dietary intervention with natural AOs in vegetables and plant oil on DNA damage in diabetics (IDDM-T2 and NIDDM), IFG and healthy subjects for 8 weeks.

3 Materials and methods

3.1 Study design

The study was a randomised intervention trial and was approved by the ethics committee of the city of Vienna. The participants were recruited by three doctors of the health centre Vienna South according to inclusion and exclusion criteria, through supplementory flyers distributed together with the journal "Diabetes – Info" of the Austrian diabetes association (ÖDV) and through a presentation at the "Diabetes Day".

All interested subjects were invited to a presentation to learn details on the study schedule, the food and plant oil intake and their rights. In total ten events took place in the health centre Vienna South and two presentations at the ÖDV within a period from January to June 2010. The subjects gave their written consent, when they wanted to participate in the study. 130 subjects participated in the general presentation. 21 subjects received information via telephone. 120 subjects out of them gave their written consent. Altogether 111 subjects started the study.

The subjects were randomly assigned to control or intervention group. The control group received information about a healthy diet, the intervention group was requested to consume 300 g of vegetables and 25 g of a plant oil per day. (Tab. 4).

Tab. 4: Study design

	Intervention group	Information group
IDDM-T2	24	11
NIDDM	30	11
IFG	9	3
Healthy	5	6

IDDM-T2 = insulin dependent T2DM ; NIDDM = non-insulin dependent T2 ; IFG = impaired fasting glucose

3.1.1 Time schedule

The day before intervention started, blood was collected for the first time (T0) from the fasting subjects. The intervention phase lasted in total eight weeks. During this time the subjects were requested to maintain their normal lifestyle. All subjects, who were in the intervention group, consumed 300 g vegetables and 25 g plant oil daily. More blood

samples were collected four weeks (T1) and eight weeks (T2) after the start of the study. A return to usual diet period of eight weeks followed and at the end blood samples were taken the last time at week 16 (T3). The body weight, body height and waist circumference were recorded at all times. In addition to the blood samples the subjects were requested to fill in 24-h recalls, food frequency questionnaires (FFQ) and quality of life questionnaires (QLQ) (Tab. 5).

Tab. 5: Intervention design

Intervention		Return to usual diet	
↑	↑	↑	↑
week 0 (T0)	week 4 (T1)	week 8 (T2)	week 16 (T3)
At all 4 time points			
Blood samplings			
Anthropometrical measurements			
Blood pressure			
Questionnaires (24-h recall, FFQ, QLQ)			

Study group

111 subjects were included in the study according to the defined inclusion and exclusion criteria. The subjects with T2DM were divided to NIDDM and IDDM-T2. All subjects with NIDDM were treated with oral antidiabetic drugs. Insulin and partly antidiabetic drugs were taken from IDDM-T2 subjects.

During the study it turned out that some of the planned healthy subjects met the criteria for IFG. The pre-diabetes state affected 12 subjects. Therefore the study group was divided into 4 states of health. A total of 99 subjects completed the study (Tab. 6).

Tab. 6: Description of the study collective

	Female	Male	Age in years (mean ± SD)	BMI in kg/m^2 (mean ± SD)
IDDM-T2	20	16	65,0 ± 7,68	33,8 ± 6,46
NIDDM	22	18	65,2 ± 7,38	33,3 ± 6,15
IFG	10	1	61,0 ± 6,86	27,7 ± 4,14
Healthy	6	6	63,8 ± 6,26	29,5 ± 3,42

SD = standard derivation

3.1.2 **Inclusion and exclusion criteria**

The inclusion and exclusion criteria for the participants of the study are listed in the tables 7 and 8.

Tab. 7: Inclusion criteria

*Men and women with **T2DM***

- *40 – 80 years of age*
- *Treatment with oral antidiabetics or insulin*

*For at least **4 weeks prior starting** the study:*

- *Constant medication concerning glucose, lipid and uric acid metabolism*
- *Constant dietary habits and physical activity*
- *Steady body weight*

***HbA1c** ≤ 9,5 % (fluctuation < 10 %)*

***Total cholesterol** < 300 mg/dl (with or without medication)*

***Serum-triglycerides** < 500 mg/dl (with or without medication)*

***Creatinine** < 2,5 mg/dl*

***Medication** of the **non-insulin-dependent** group: Metformin, DPP-IV-Inhibitors, Sulfonylureas, GLP-1 Mimetics*

***Medication** of the **insulin-dependent** group: insulin or insulin and metformin*

Tab. 8: Exclusion criteria

*Men and women with **T1DM***

Patients younger than 40 and older than 80 years

***Smoker**s*

Pregnant** and **breastfeeding women

Participation** in **another clinical trial

***New medication** or **change** of medication concerning glucose, lipid and uric acid metabolism **4 weeks before** starting the study*

*Intake of **fish oil capsules** and other **fatty acids***

*During or **prior** starting the study intentions of **changing***

- *Eating habits*
- *Physical activities*
- *Body weight*

***Cardiovascular diseases**, defined by NYHA- classification ≥ III*

***Liver diseases** (transaminase-threshold higher than ≥ 2,5 times)*

***Chronic renal failure** (dialysis patients or creatinine > 2,5 mg/dl)*

Organ transplantation

***Gastrointestinal malabsorption** (pancreatic insufficiency, steatorrhea, short bowel syndrome)*

Systematic steroids

***Drug and alcohol abuse** (≥ 80 g/d); methadone intake during the last 2 years*

Cancer, HIV

***Glitazone** intake*

3.1.3 **Vegetables and plant oil**

For the study a walnut oil, which was rich in PUFAs and α-tocopherols was used.

The frozen vegetables were distributed by Iglo Austria. The main focus was on green vegetables, which are rich sources of folic acid. In table 9 is shown a list of vegetables available for the subjects during the intervention.

Tab. 9: Vegetables

Broccoli	Courgette
Brussels sprouts	Green beans
Cabbage turnip	Maize
Carrots	Peas
Cauliflower	Soya beans
Cos lettuce	Spinach

Recipe suggestions for the subjects were provided from us.

3.2 **Principles of method**

The comet assay is a widely used method for the detection of DNA damage and gives information the number of single and double strand breaks. The cells are embedded in agarose on a microscope slide. Then they are lysed for removing proteins, membranes, cytoplasm and nucleoplasmic constituents. The DNA is left as a nucleotide with a compact structure, but without histones. Under alkaline conditions the DNA unwinds and is electrophoresed later. In the electrophoretic field damaged DNA migrates faster as the compact and undamaged DNA and forms a comet like image. After staining, the comets can be evaluated with a fluorescence microscope (Fig. 4). For the measurement of different forms of damage, nucleotide are incubated with lesion-specific repair enzymes. These enzymes convert oxidised bases to strand breaks. Endo III allows to detect oxidised pyrimidines and FPG recognises oxidised purines [COLLINS, 2004].

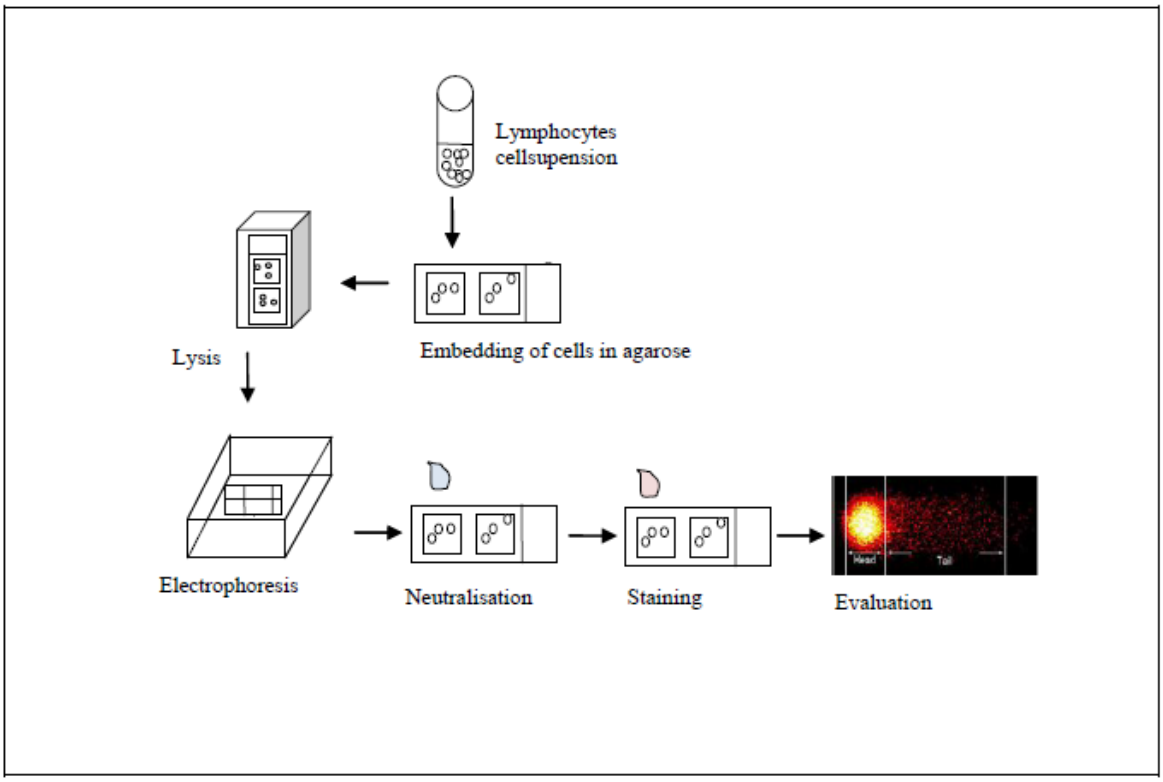


Fig. 4: Scheme of the comet assay

3.2.1 **Chemicals and working equipment**

3.2.1.1 **Chemicals and Reagents**

Tab. 10: Chemicals and Reagents used in the Comet assay

Substance	Supplier	Product number
Bovine serum albumin (BSA)	*Sigma*	A2153
Dulbeccos`s Phosphate Buffered Saline (PBS)	*Sigma*	D8537
Ethidiumbromid Solution 20µg/ml	*Sigma*	E1510
Ethylenediamine tetraacetic acid (EDTA)	*Sigma*	E6758
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	*Sigma*	H3375
Hydrochloric acid (concentrated HCl)		
Potassium chloride (KCl)	*Riedel-de Haen*	31248
Potassium hydroxide (KOH)	*Sigma*	60370
Sodium chloride (NaCl)	*Sigma*	71380
Sodium hydroxide (NaOH)	*Riedel-de Haen* *Sigma*	06203
Trizma Base (Tris)	*Fluka Analytical*	93350
Triton X, *t*-Octylphenoxypolyethoxyethanol	*Sigma*	X-100
Trypan blue Solution	*Invitrogen*	T10282
Ultra Pure Agarose, normal melting Agarose (NMA)	*Invitrogen*	16500
Ultra Pure LMP Agarose, low melting Agarose (LMA)	*Invitrogen*	16520

3.2.1.2 **Equipment**

Tab. 11: Equipment used in the Comet assay

Substance	Supplier
Slides	
Coverslips 20x20 mm	
Coverslips 22x22 mm	
Centrifuge/Solid bowl centrifuge	
691 ph-Meter	*Metrohm, swiss made*
Magnetic stirrer	*Heidolph MR 3001K*
Wather bath (GFL Müller und Scher)	
Power supply (Pequlab)	
Electrophoresis chamber	
Gloves (nitril)	
Microscope (Axioskop, Zeiss,Hitachi)	
Komet 5.5, image analysis system (Kineting Imaging)	
Microwave	
6 metal plates	
Tube 50 ml (for enzymbuffer)	

3.2.1.3 **Preparation of general solutions and reagents**

All solutions were kept at 4°C or cooled down before usage.

Lysis solution (pH = 10)

	amounts per liter
2.5 M NaCl	146.1 g
0.1 M EDTA	37.2 g
10 mM Tris	1.2 g

By addition of 10 M NaOH the pH was set to 10. 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.

Neutralising buffer

	amounts per liter
0.4 M Tris Base	48.44 g

The pH was set to 7.5 by addition of concentrated HCl solution.

Enzyme reacton buffer for endonuclease III and FPG, Stock solution (10 x stock)

	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.

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)

200 mg LMA per 20 ml PBS

Normal melting agarose (NMA)

200 mg NMA in 20 ml aqua bidest

Ethidium bromide (20 µg/mL)

10 µl stock solution (10 mg/mL)

5 ml aqua bidest

3.3 Comet Assay

Outline protocol of comet assay is based on Azqueta [AZQUETA et al., 2009].

3.3.1 Slide preparation

The NMA was heated in a microwave until the agarose was dissolved and the solution was clear. The jar with the agarose was placed in a water bath at a temperature of 55°C. The slides were dipped into the agarose. Then the back side was wiped clean with a paper towel and the slides were dried at room temperature over night.

The precoated slides were stored at room temperature.

3.3.2 Lymphocyte isolation

The cell preparation tubes Heparin were inverted 8 times immediately after blood sampling and were centrifuged within two hours after blood sampling.

Before centrifugation the blood sample was remixed by inverting the tubes 5 times and centrifugated at 3100 rpm (rounds per minute) for 25 min at 22°C. Then the tubes were inverted one time. The cell suspension was transferred in another tube and filled up with PBS to 15 ml. The tubes were inverted 5 times to mix the cells and centrifugated at 1300 rpm for 15 min at 4°C . The supernatant was aspirated and PBS was added to a volume of 10 ml. The tubes were inverted 5 times again and centrifugated at 1300 rpm for 10 min at 4°C. After centrifugation the supernatant was aspirated and resuspended in 1 ml PBS. For comet assay a minimum of 240 µl cell suspension with a concentration of 10^6 cells/ml was needed.

Cell number was defined with an automated cell counter (Countess, Invitrogen). 10 µl of cell suspension and 10 µl trypan blue were mixed and 10 µl out of the mixture were transferred into a chamber slide, were living cells were counted.

3.3.3 Embedding cells in agarose

The slides precoated with NMA were used and labelled with the subjects number and the kind of treatment: “lysis”, “H2O2”, “buffer”, “FPG” and “EndoIII”.

The jar with the dissolved LMA was placed in the water bath at 37°C.

Metal plates were chilled in the cool lab before using. The microscope slides were placed on the cooled plates.

30 µl of cell suspension with a concentration of 1 x 10^6 cells/ml was mixed with 140 µl of LMA. Quickly two drops each with 70 µl were placed on one slide and immediately

covered with cover slips. The slides were left in the cool lab for 5 minutes. During this time the agarose set.

3.3.4 **Treatment with H_2O_2**

The cover slips were removed gently and the slides were placed in a jar filled with 100 µM H_2O_2-solution for 5 minutes at 4°C. Afterwards the slides were dipped in PBS shortly to wash remove the H_2O_2.

3.3.5 **Lysis**

The lysis solution was filled into jars. Cover slips were removed and all slides were placed into the jars. For the slides treated with H_2O_2 a separate jar was used. They were left in the cool lab for more than 1 h at 4°C.

3.3.6 **Enzyme treatment**

Before treatment the slides “buffer”, “FPG” and “Endo III” were washed 3 times with enzyme buffer each time for 5 minutes at 4°C. The buffer was dabbed off with a paper towel. 50 µl of buffer (as control) or FPG/Endo III solution was placed on the gels and covered with cover slips. The slides were put into a moist box and were incubated at 37°C for 30 minutes. The cover slips were removed after the end of the incubation period.

The slides labelled with “lysis” and “H2O2” remained in the lysis solution until the end of the incubation period.

3.3.7 **Alkaline treatment**

The electrophoresis buffer was cooled before use. All slides were placed side by side with two in a row in the electrophoresis tank. Gaps in a row were filled up with a blank slide. The electrophoresis buffer was added until the slides were completely covered. Incubation period was 20 minutes.

3.3.8 **Electrophoresis**

The electrophoresis was running 30 min at 25 V and around 300 mA. When 25 V was not reached or the current was too high, some buffer was removed.

3.3.9 **Neutralisation**

The slides were removed from the electrophoresis and washed with neutralising buffer in a jar at 4°C for 5 min followed by 5 min with water. Afterwards excessive water was

dabbed off and the slides were left at room temperature over night in the dark for drying.

3.3.10 **Staining**

30 µl ethidium bromide was placed on the gel and covered with a cover slip. The stained slides were kept in the dark until they were scored.

3.3.11 **Quantitation**

For the evaluation of DNA damage Komet 5.5 image analysis software, which was linked to a fluorescent microscope was used. For each sample two gels with each 50 cells were randomly scored. The percentage of DNA in the tail (% tail DNA) was determined and the mean was calculated.

3.3.12 **Statistical analysis**

All data are expressed as mean ± standard derivation (SD). The data were analysed with SPSS 17.0 for Windows. The normality test was performed by the Kolmogorov-Smirnov test. For baseline comparisons unpaired t-test and the one way ANOVA were used. A repeated-measures analysis of variance was used to measure the effect of the intervention. Statistical differences were considered to be significant at a value of $p \leq 0.05$.

4 Results and discussion

4.1 Baseline Comparisons

4.1.1 Clinical characteristics of the study population at baseline

The control and intervention group did not differ in body mass index (BMI), HbA1c, triglycerides, fasting plasma glucose and diabetes duration at baseline. Only age was slightly, but significantly greater in the intervention group when compared to the control group (Tab. 12).

Tab. 12: Clinical characteristics of the control and the intervention group at baseline

	Control	Intervention
N	31	68
Age (years)	61.65 ± 6.66	65.76 ± 7.29*
BMI (kg/m^2)	32.21 ± 7.03	32.50 ± 5.71
Diabetes duration (years)	12.59 ± 9.21	15.13 ± 9.98
HbA1c (%)	7.01 ± 1.05	7.23 ± 1.08
Triglycerides (mmol/l)	1.45 ± 0.74	1.49 ± 0.72
Fasting plasma glucose (mmol/l)	7.41 ± 2.07	8.12 ± 2.34

Data are means ± SD; * indicates significant differences ($p \leq 0.05$)

4.1.2 Parameters of oxidative DNA damage at baseline of healthy, IFG- and diabetic subjects

The levels of DNA damage, oxidised purines and pyrimidines and resistance to H_2O_2 at baseline were not significantly different between IDDM-T2DM, NIDDM, IFG- and healthy subjects (Tab. 13).

Tab. 13: Oxidative DNA damage in control and intervention group separated at baseline

	Control	Intervention
Lysis		
IDDM-T2	4.48 ± 1.08	6.59 ± 2.99*
NIDDM	4.36 ± 0.87	5.25 ± 1.47
IFG	5.02 ± 0.65	5.77 ± 2.03
Healthy	5.32 ± 1.01	4.11 ± 1.23
H_2O_2		
IDDM-T2	20.62 ± 7.66	23.78 ± 9.88
NIDDM	21.16 ± 6.42	23.50 ± 11.22
IFG	23.73 ± 12.03	20.44 ± 12.05
Healthy	20.73 ± 5.94	25.16 ± 9.85
FPG		
IDDM-T2	4.42 ± 4.14	4.35 ± 2.22
NIDDM	3.68 ± 2.18	3.46 ± 2.56
IFG	3.72 ± 2.81	2.09 ± 1.24
Healthy	3.00 ± 1.26	2.70 ± 1.11
Endo III		
IDDM-T2	1.81 ± 1.05	1.51 ± 1.34
NIDDM	1.11 ± 0.80	1.20 ± 1.00
IFG	1.14 ± 0.44	1.31 ± 1.18
Healthy	0.71 ± 0.32	2.01 ± 2.54

Data are means ± SD; * indicates significant differences

There were no significant differences between the control and intervention group in the levels of DNA damage, H_2O_2-induced oxidative DNA damage, FPG- and Endo III-sensitive sites at baseline. Only for subjects with IDDM-T2 significant differences between the two groups were observed at baseline.

The subjects with diabetes and IFG showed slightly, but not significantly higher levels of DNA damage when compared to healthy controls. Levels of oxidised bases were not significantly greater in subjects with diabetes in comparison to the healthy subjects.

Several studies showed that oxidative DNA damage in subjects with diabetes was significantly elevated when compared to healthy individuals [COLLINS et al., 1998; LODOVICI et al., 2008; SONG et al., 2007]. FPG-sensitive oxidative DNA damage was reported to be significantly increased in subjects with T2DM in comparison to healthy controls [DINÇER et al., 2002; PITOZZI et al., 2003].

Our results indicate that patients with T2DM do not have significantly greater DNA damage than healthy individuals. Our findings are in agreement with Hannon-Fletcher et al. [HANNON-FLETCHER et al., 2000] and Ibarra-Costilla et al. [IBARRA-COSTILLA et al., 2010], who reported no significant differences of DNA damage in subjects with diabetes when compared to healthy controls. This might be due to the good glycaemic control of our T2DM subjects who were metabolically stable.

4.1.3 Impact of glycamic control (HbA1c and fasting plasma glucose) on oxidative DNA damage

There were no significant differences between good (HbA1c $\leq$ 7.9%) and poor (HbA1c $\geq$ 8.0%) glycaemic controlled subjects of the whole study population in DNA damage, resistance to H_2O_2-induced DNA damage and oxidised purines and pyrimidines at baseline. The better controlled IDDM-T2 subjects showed significantly higher resistance to H_2O_2-induced DNA damage in comparison to the poor controlled IDDM-T2 subjects at baseline ($p \leq 0.05$; Fig. 5). DNA damage in the IDDM-T2 subjects with good control was not significantly lower than in poor controlled IDDM-T2 subjects.

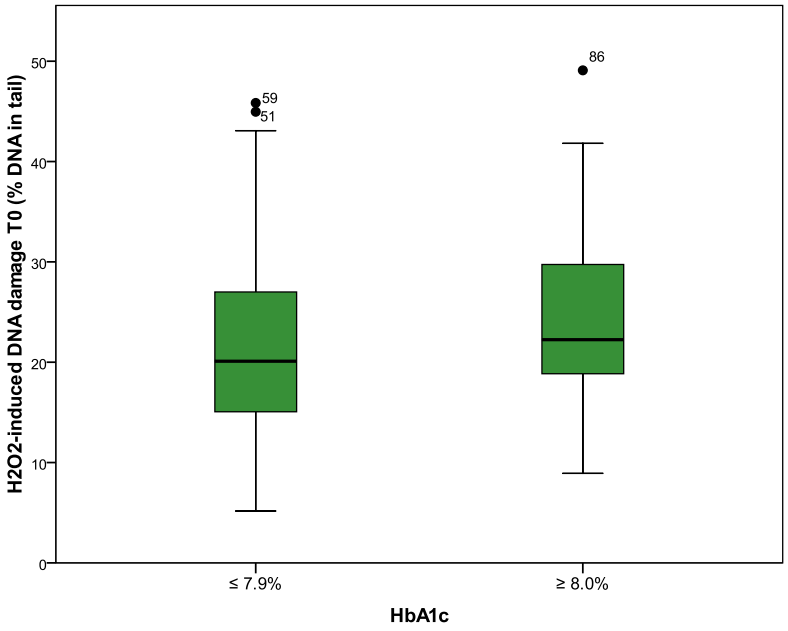


Fig. 5: H2O2-induced DNA damage of IDDM-T2 subjects with good and poor glycaemic control at baseline ($p \leq 0.05$).

Considering the total study group subjects with high fasting plasma glucose (≥ 6.21 mmol/l) showed significantly higher levels of oxidised purines than subjects with normal fasting plasma glucose (≤ 6.2 mmol/) (Fig. 6).

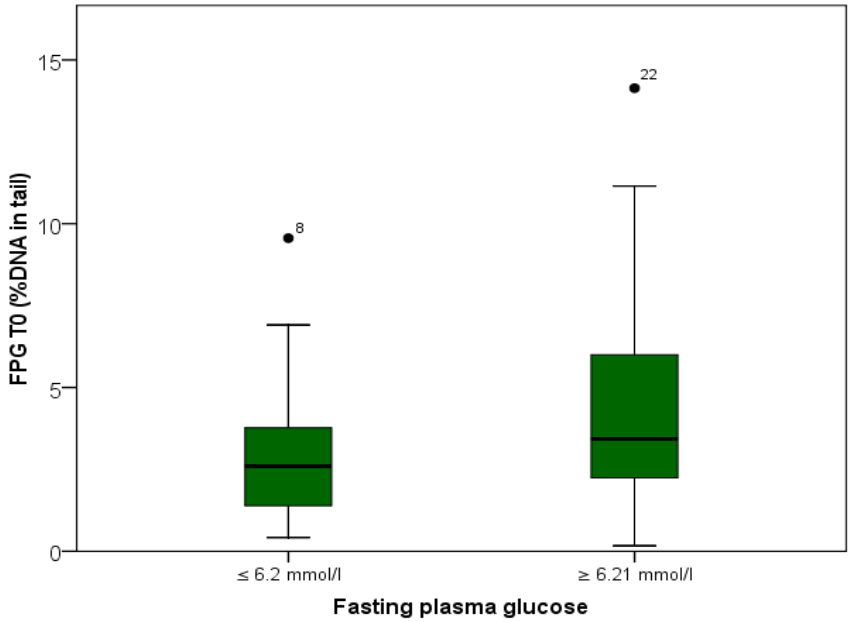


Fig. 6: Oxidised purines in subjects with normal and elevated levels of fasting plasma glucose in the total group at baseline ($p \leq 0.05$).

No significant differences in DNA-damage, resistance to H_2O_2, FPG- and Endo III-sensitive sites were seen in IDDM-T2 subjects with high or low levels of fasting plasma glucose at baseline.

Our results indicate that IDDM-T2 subjects with poor glycaemic control have lower resistance to H_2O_2-induced DNA damage when compared with good controlled IDDM-T2 subjects. The antioxidant capacity may be lower in patients with poor glycaemic control than in good controlled IDDM-T2 subjects [CHOI et al., 2005; LODOVICI et al., 2008]. In accordance to our study, Choi et al. reported that oxidative DNA damage was significantly increased in T2DM subjects with poor glycaemic control and lower status of plasma ascorbic acid than in subjects with higher concentrations of plasma ascorbic acid [CHOI et al., 2005]. Lodovici et al. demonstrated similar findings: poor glycaemic controlled T2DM subjects have a slightly lower plasma antioxidant status and greater levels of DNA damage in comparison with healthy controls [LODOVICI et al., 2008]. Furthermore, Collins et al. found in T1DM subjects with poor glycaemic control higher levels of oxidative DNA damage [COLLINS et al., 1998]. Dinçer et al. evaluated in T2DM subjects higher levels of FPG-sensitive oxidative DNA damage. They were significantly increased in diabetics with poor glycaemic control in comparison to good controlled subjects [DINÇER et al., 2002].

Collins et al. demonstrated in T1DM subjects, that the amount of FPG-sensitive DNA damage is significantly increased in subjects with higher glucose levels in comparison to subjects with normal levels [COLLINS et al., 1998]. Similarly, Lodovici et al. found also an association between higher FPG-sensitive DNA damage and higher concentration of plasma glucose in subjects with T2DM [LODOVICI et al., 2008].

In our study we showed that subjects with higher levels of fasting plasma glucose have greater levels of DNA damage. Our findings support the hypothesis that hyperglycemia results in increased oxidative stress leading to elevated oxidative DNA damage.

4.1.4 **Impact of age, BMI, diabetes duration and triglyceride concentrations**

At baseline, there were no associations between oxidative DNA damage and age, BMI, diabetes duration and triglyceride concentrations in the whole study population.

When subjects were divided into groups regarding to their state of health, there was also no impact of age, BMI, diabetes duration and triglyceride concentrations on oxidative DNA damage.

Ibarra-Costilla et al. found no significant differences on DNA damage in subjects with long-term T2DM (> 5 years) when compared to the healthy control group [IBARRA-COSTILLA et al., 2010]. A study performed by Song et al. reported that subjects with IFG showed significantly lower levels of DNA damage when compared to newly diagnosed T2DM subjects [SONG et al., 2007].

Piperakis et al. also demonstrated that oxidative DNA damage is affected by age. Lower resistance to H_2O_2-induced DNA damage was observed in the older healthy subjects (aged between 55 - 60 years) when compared to younger healthy subjects (aged 20 - 25 years) [PIPERAKIS et al., 1998]. Bagatini et al. found no association between DNA damage and age in subjects with T2DM at baseline [BAGATINI et al., 2008].

4.2 Effect of the intervention

The following chapters describe the effect of the intervention in the whole study group and especially in subjects with IDDM-T2. The impact of HbA1, fasting plasma glucose and triglycerides and the association of age on oxidative DNA damage during the intervention are also shown. Furthermore, results of dietary and supplementation intervention studies are discussed and compared with findings of our study.

4.2.1 Effect of the intervention in control and intervention group

The results showed no significant changes in levels of DNA strand breaks, resistance to H_2O_2, oxidised purines and pyrimidines in the control group during the whole study period.

In the intervention group the levels of DNA damage changed significantly during the intervention period. The decrease in DNA damage was 17.63% after 4 weeks in comparison to baseline, but these results were not significant. The high SD at baseline in the intervention group was mainly responsible for the insignificant changes. After 8 weeks of intervention, a significant decrease of 20.77% in DNA damage was seen when compared to baseline (Fig. 7).

The intervention group also displayed positive effects in the resistance to H_2O_2-induced DNA damage, but the differences were not statistically significant (T0: 23.32 ± 10.59, T1: 23.11 ± 7.88, T2: 21.76 ± 7.45). No effects were seen in the levels of FPG- and Endo III-sensitive oxidative DNA damage.

The results showed, that the levels of oxidative DNA damage were slightly higher at T3 (after return to normal diet) in comparison to T2 (after 8 weeks of intervention). DNA damage was not significantly higher at baseline when compared to T3.

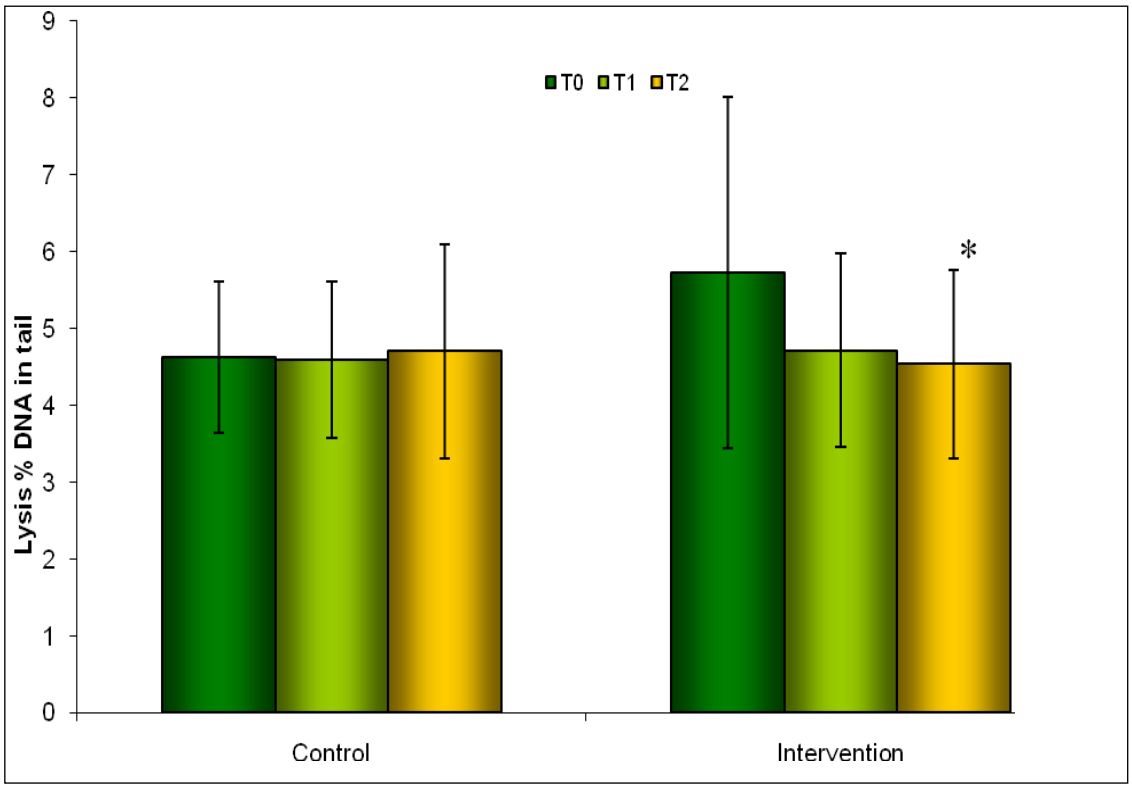


Fig. 7: Levels of DNA damage were significantly decreased in the intervention group but remained constant in the control group ($p \leq 0.05$; * indicates a significant difference from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

A number of studies have evaluated the effects of antioxidants in vegetables or as supplements on DNA damage. Depending on the experimental design, the intervention showed beneficial or no effects in the levels of DNA damage or resistance to DNA damage when treated with H_2O_2.

Our findings are in agreement with those of Pool-Zobel et al. Their study showed a significant decrease in oxidative DNA damage after consumption of carrot juice for 2 weeks [POOL-ZOBEL et al., 1997]. However, Riso et al. and Porrini et al. reported a significant increase in the resistance to H_2O_2-induced DNA damage (treated with 500 µmol/l H_2O_2-solution) [RISO et al., 1999; PORRINI et al., 2002]. On the contrary, no effects of a daily intervention in healthy subjects with 600 g of fruits and vegetables for 24 days on oxidative DNA damage, resistance to H_2O_2-induced DNA damage (treated with 150µmol/l), FPG- and Endo III-sensitive DNA damage were seen [MØLLER et al., 2003]. A dietary intervention with carotenoid-rich foods or supplementation with carotenoids for 3 weeks had no effect on oxidative DNA damage and resistance to H_2O_2-induced DNA damage (treated with 100 µmol/l H_2O_2) [ASTLEY et al., 2004]. A study performed by Gill et al. showed after a dietary intervention with cruciferous and

leguminous sprouts for 2 weeks significantly increased resistance to H_2O_2-induced DNA damage (75 µmol/l H_2O_2-solution). But the intervention had no effect on DNA damage and oxidised purines [GILL et al., 2004]. After consumption of 300 g of Brussels sprouts per day for 6 days levels of oxidative DNA damage were decreased. The results showed significantly reduced levels of oxidised pyrimidines and higher resistance to H_2O_2-induced DNA damage [HOELZL et al., 2008].

A long term supplementation with multivitamin tablets containing Vitamin E, C and β-carotene showed no effects on DNA strand breaks. But after 20 weeks increased resistance to H_2O_2-induced DNA damage and lower levels of Endo III-sensitive sites were observed [DUTHIE et al., 1996]. After intake of 60 mg or 6 g Vitamin C per day for 2 weeks in a cross-over study (6 weeks between the treatment periods) no effects on DNA damage and H_2O_2 resistance were observed [ANDERSON et al., 1997].

In conclusion, our results show that the daily consumption of 300 g vegetables and 25 g of plant oil significantly reduce oxidative DNA damage in T2DM subjects. In our study we demonstrated, that an intervention of 4 weeks with different vegetables and plant oil showed a reduction, which was due to the high SD at baseline not significant. After 8 weeks of intervention the levels of DNA damage were reduced significantly. So far conflicting results on vegetable and supplementation trials have been reported. One explanation for that could be, that our intervention was over a longer time period (8 weeks of intervention) than the other trials. Another reason is the study group itself. Most of the studies so far were performed with healthy subjects. We investigated effects on diabetics, which are less studied in this respect so far. After treatment with H_2O_2, the resistance to DNA damage was improved, but statistically not significant. The reason could be that the concentration of 100 µmol/l H_2O_2-solution was not high enough for greater impact on lymphocyte DNA damage. Other studies have used H2O2-solutions with concentrations up to 500 µmol/l.

4.2.2 **Effect of the intervention on IDDM-T2**

Table 14 shows, that IDDM-T2 subjects of the intervention group have higher levels of DNA damage than the IDDM-T2 subjects of the control group at baseline. In the control group levels of oxidative DNA damage did not change during 8 weeks. The IDDM-T2 subjects in the intervention group displayed an insignificant decrease of 21.70% in DNA strand breaks after 4 weeks and a significant reduction of 27.01% after 8 weeks with respect to baseline (Fig. 8).

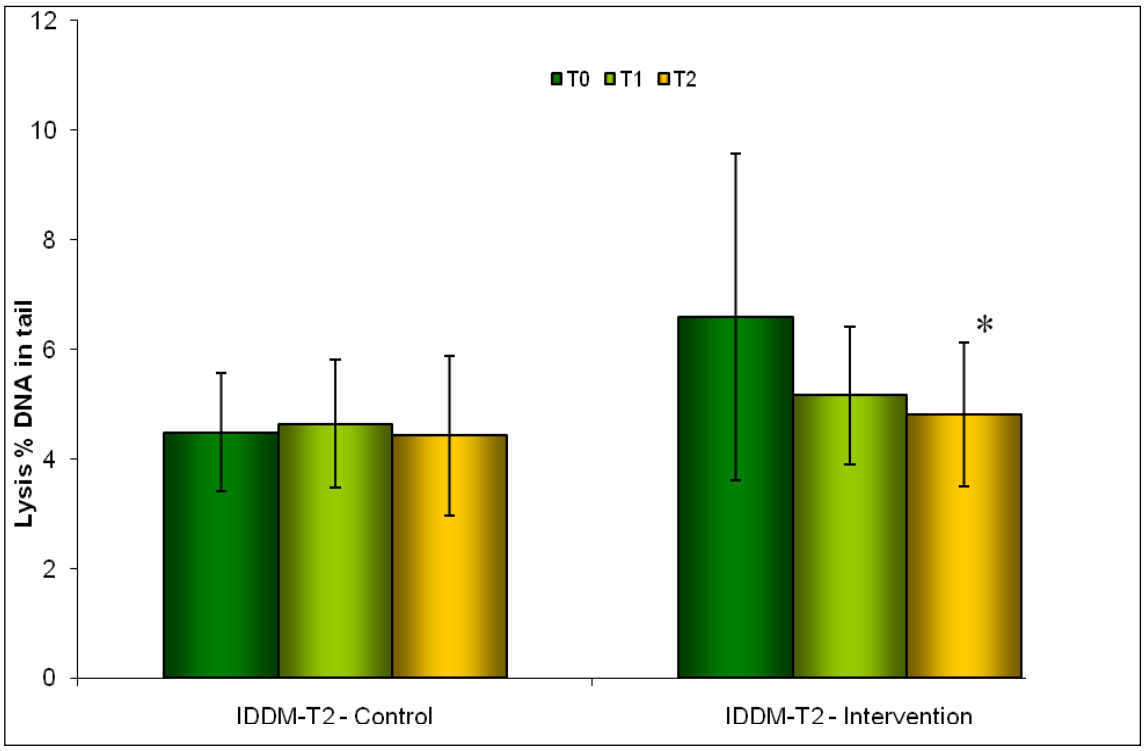


Fig. 8: The levels of DNA damage were significantly decreased after 8 weeks of intervention in the IDDM-T2 subjects but remained constant in the control group ($p \leq 0,05$; * indicates significant differences from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

The resistance of oxidative DNA damage to H_2O_2 was slightly but not significantly increased in subjects with IDDM-T2 after 8 weeks of intervention (T0: 23.78 ± 9.88, T1: 23.13 ± 6.39, T2: 21.56 ± 5.33). Decreased levels of oxidised purines were also seen in IDDM-T2 patients, but the changes were not significant (T0: 4.61 ± 2.24, T1: 4.39 ± 2.80, T2: 3.55 ± 2.71). No changes in oxidised pyrimidines were observed (T0: 1.83 ± 1.53, T1: 1.55 ± 0.94, T2: 1.66 ± 2.22).

Astley et al. [ASTLEY et al., 1999] and Sampson et al. [SAMPSON et al., 2001] did not see any effect of an 8 week supplementation with α–tocopherol (400 IU) on DNA damage and H_2O_2 resistance in subjects with T1DM or T2DM. On the other hand, an

antioxidant supplementation with high amounts of α–tocopherol (900 IU) daily for 12 weeks showed a significant decrease of DNA damage in patients with T2DM and T1DM [ŞARDAŞ et al., 2001]. These results from the long-term studies mentioned above show conflicting effects of single supplementation of α-tocopherols in subjects with diabetes. Only one study indicate the beneficial effects on DNA damage in T2DM subjects with high dosage of α-tocopherols over a long time period.

After 2 weeks of a flavonol-rich diet (supplemented with 76-110 mg of flavonols) among subjects with T2DM, significantly greater resistance to H_2O_2-induced DNA damage (100 µmol/l H_2O_2-solution) was seen, but there were no effects on Endo III sensitive oxidative DNA damage [LEAN et al., 1999]. These results demonstrate, that also a short term intervention with flavonol supplements has protective effects on DNA damage in subjects with T2DM and improves antioxidant capacity.

In summary, we found in IDDM-T2 subjects high reductions of about 22% (T1) and 27% (T2) in the levels of DNA damage when compared to baseline. We demonstrated that natural AOs from vegetables and plant oil have the potential to reduce oxidative DNA damage significantly in comparison to the reported supplementation studies with diabetics. We could show that dietary intake of different vegetables and plant oil has more beneficial influence on DNA damage than supplementation trials with the same time period. Therefore it is possible to decrease DNA damage and probably also diabetic complications in T2DM subjects by improving the usual diet.

4.2.3 **The impact of HbA1c on DNA damage**

Subjects of the intervention group with a HbA1c ≤ 7.9% had significantly higher levels of DNA damage in comparison to controls at baseline. Subjects with HbA1c ≤ 7.9% (n = 49) showed a not significant decrease in DNA damage by 13.63% after 4 weeks and a significant reduction of 17.68% after 8 weeks of intervention (Fig. 9). Subjects with HbA1c ≥ 8.0% (n = 19) showed no significant changes during the intervention (control and intervention group). But there was a clear trend to reduced levels of DNA damage in the intervention group (T0: 6.50 ± 3.38, T1: 4.78 ± 0.90, T2: 4.71 ± 1.27). The reason why we did not find any significant changes might be the lower number of subjects with HbA1c ≥ 8.0% or the high SD at baseline.

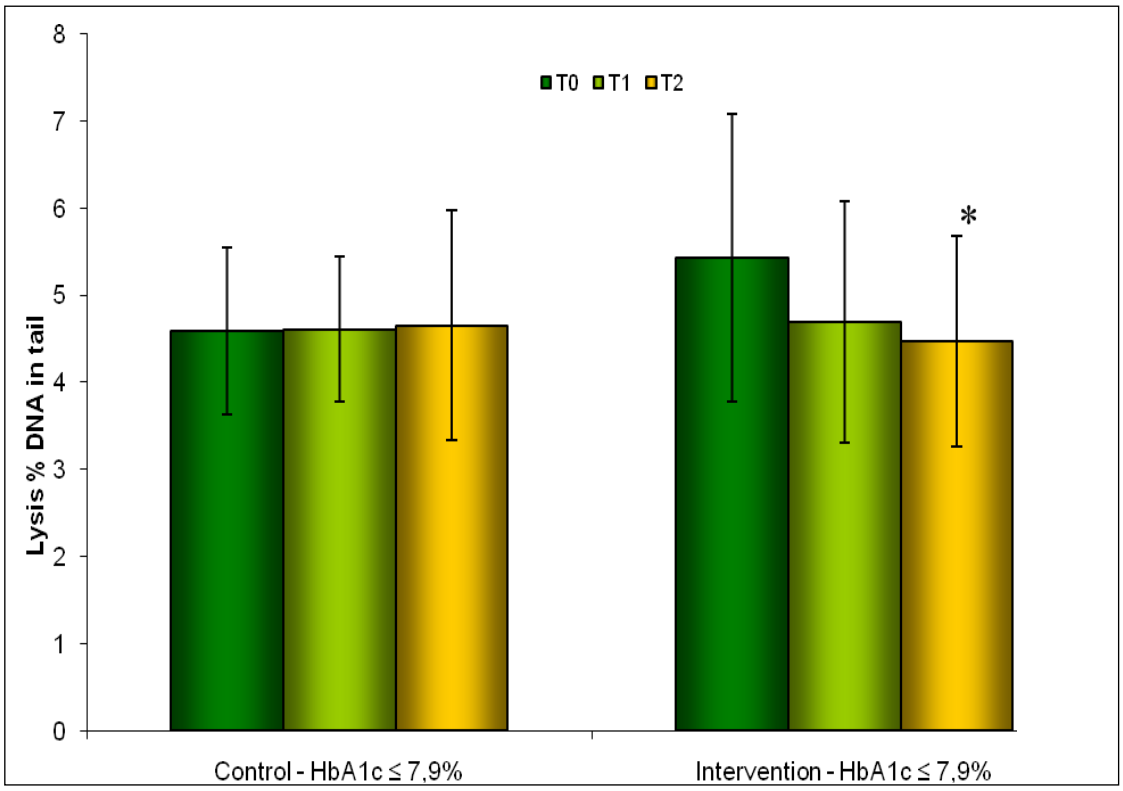


Fig. 9: Subjects of the intervention group with HbA1c ≤ 7.9% showed significant decreases of DNA damage in comparison to baseline ($p \leq 0.05$; * indicates a significant difference from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

No significant differences in DNA damage between T3 (after return to normal diet) and T0, T1 or T2 were observed, the damaged DNA was not significantly higher at baseline when compared to T3.

Subjects with HbA1c ≥ 8.0% showed an insignificant higher resistance to H_2O_2-induced DNA damage during the intervention (T0: 25.59 ± 11.14, T1: 23.65 ± 6.95, 21.84 ± 6.32). No changes were seen in Endo III- and FPG-sensitive oxidative DNA damage.

4.2.4 The impact of HbA1c on DNA damage in IDDM-T2 subjects

IDDM-T2 subjects with HbA1c ≥ 8.0% showed higher levels of DNA damage at baseline in comparison to subjects with HbA1c ≤ 7.9%, but the results were not significant. DNA damage was reduced in both groups after 8 weeks of intervention. The decrease in the levels of DNA damage was 6.9% after 4 weeks and after 8 weeks levels were significantly reduced by 20.71% in the better controlled IDDM-T2 subjects. The weaker controlled subjects with IDDM-T2 showed also a high reduction in DNA damage after 4 weeks (-28.25%) and 8 weeks (-29.73%) when compared to baseline,

but it was not significant. The high SD in the group of IDDM-T2 with HbA1c ≥ 8.0% at baseline was mainly responsible for the only insignificant changes (Fig. 10).

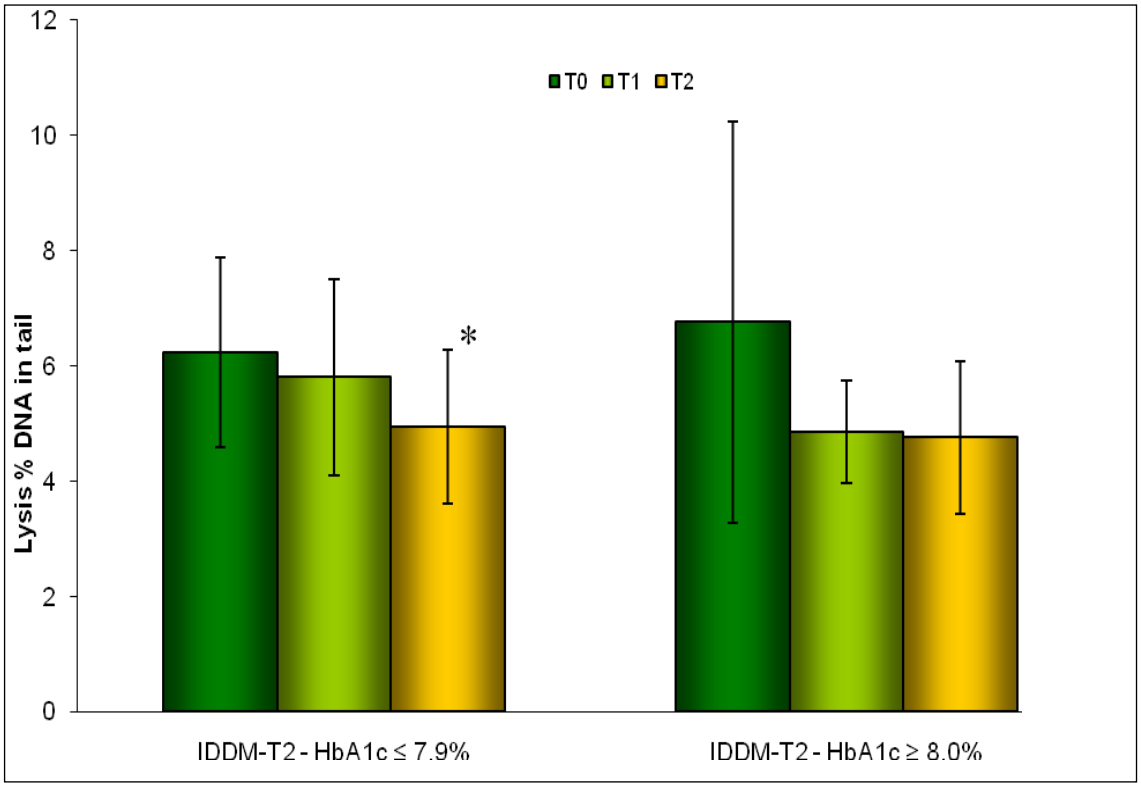


Fig. 10: IDDM-T2 subjects with HbA1c ≤ 7.9% showed significant decreases of DNA damage in comparison to baseline ($p \leq 0.05$; * indicates a significant difference from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

IDDM-T2 subjects with HbA1c ≥ 8.0% displayed positive effects in the resistance to H_2O_2-induced DNA damage, but the changes were not significant (T0: 25.66 ± 10.35, T1: 23.63 ± 6.49, T2: 21.69 ± 5.58). FPG-sensitive oxidative DNA damage in IDDM-T2 patients with HbA1c ≤ 7.9% was not significantly reduced after 4 weeks in comparison to baseline, but after 8 weeks it was slightly higher than at week 4 (T0: 4.35 ± 2.27, T1: 3.15 ± 2.47, T2: 4.03 ± 2.31). No significant changes in the levels of Endo III sensitive sites were seen in the group with better and in weaker controlled IDDM-T2 subjects during the intervention.

Our results confirm, that vegetables and plant oils have positive effects on oxidative DNA damage when considering the levels of HbA1c in IDDM-T2 subjects. A decrease in DNA damage was seen in the group with lower and higher HbA1c levels after 4 and 8 weeks. Although the reduction in the levels of DNA damage was higher in IDDM-T2 subjects with HbA1c ≥ 8.0%, the results were not significant. At baseline this group was

very heterogenous with a high SD, but the group became homogenous during the intervention. So far only Astley et al. measured the correlation between HbA1c and DNA damage in T1DM, but found no significant relationship during an 8 week intervention with α-tocopherol [ASTLEY et al., 1999].

4.2.5 The link between fasting plasma glucose and DNA damage

During the intervention period, the levels of oxidative DNA damage tended to decrease after 8 weeks (- 18.18%) in subjects with concentrations of fasting plasma glucose ≤ 6.2 mmol/l ($p \leq 0.15$; T0: 5.50 ± 1.86, T1: 4.69 ± 1.42, T2: 4.50 ± 1.52). Subjects with greater levels of fasting plasma glucose (≥ 6.21 mmol/l) displayed an insignificant reduction in DNA damage (T0: 5.87 ± 2.47, T1: 4.73 ± 1.24, T2: 4.55 ± 1.12).

The intervention showed no effect on the resistance to H_2O_2-induced DNA damage and the amount of oxidised purines and pyrimidines.

There were no significant changes after the return to normal diet in the amounts of DNA damage at week 16 in comparison to baseline and any measure during the intervention period for subjects with lower (T3: 4.73 ±1.55) and higher (T3: 4.69 ± 1.10) fasting plasma glucose concentrations.

Our results indicate that the intervention in the total study group with lower fasting plasma glucose had more beneficial effects on the levels of DNA damage.

4.2.6 The link between fasting plasma glucose and DNA damage in IDDM-T2 subjects

In IDDM-T2 subjects of the intervention group with higher fasting plasma glucose (≥ 6.21 mmol/l) levels of DNA damage tended to decrease after 8 weeks with respect to baseline ($p \leq 0.15$; T0: 6.87 ± 0.98, T2: 5.20 ± 2.48). The reduction was about 25%, but this result was not significant. No effects were seen in the group with lower and higher fasting plasma glucose concentrations on H_2O_2-induced, FPG- and Endo III-sensitive DNA damage.

In conclusion, we showed with our dietary intervention that IDDM-T2 subjects with higher fasting plasma glucose have a higher response to the intervention and more beneficial effects in the levels of damaged DNA. One explanation could be the reduction of oxidative stress because of the daily consumption of vegetables and plant oil rich in AOs. Another reason might be the positive effect of the intervention on glycaemic control in subjects with T2DM.

4.2.7 **The association between triglycerides and DNA damage**

Independent of higher or lower than 1.2 mmol/l triglyceride levels, oxidative DNA damage was reduced, although it was not significant. The group with ≤ 1.2 mmol/l triglycerides displayed a higher decrease in DNA damage after 8 weeks (-24.75%) of intervention when compared to baseline. A reduction of DNA damage was also seen in the group with higher triglyceride levels (≥ 1.3 mmol/l) after 4 weeks (-12.52%) and 8 weeks (-19.24%) in comparison to baseline. But these results were not significant due to the high SD in both groups at baseline (Fig. 11).

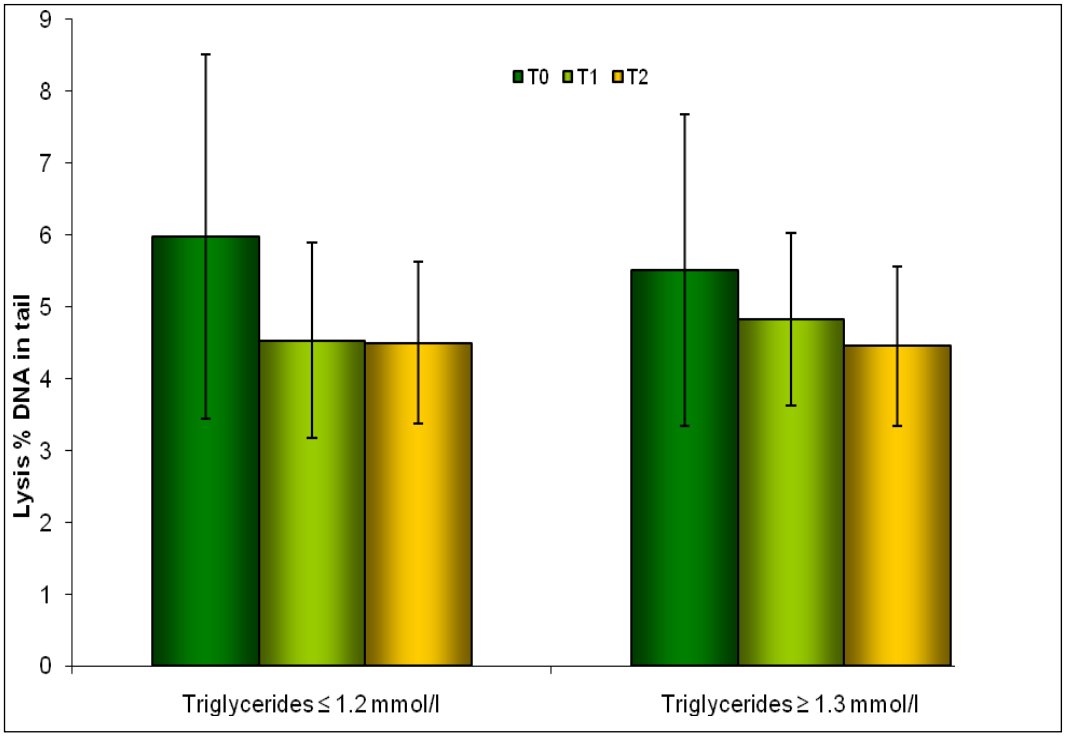


Fig. 11: Subjects with high and low levels of triglycerides showed a reduction in DNA damage. These results were not significant (p < 0.15; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

The intervention showed no significant changes in the resistance to H_2O_2-induced DNA damage, oxidised purines and oxidised pyrimidines at any time point of measure.

No significant effects of DNA damage between T3 (after return to normal diet) and T0, T1 or T2 were seen between.

Our results showed high reductions in the levels of oxidative DNA damage during the intervention period when considering triglyceride levels. Decreased levels of oxidative DNA damage were observed in both groups. The subjects with lower levels of triglycerides showed a higher reduction in DNA damage than subjects with higher triglyceride levels. Although the reductions were high, the results were not significant.

At baseline the SD was very high in both groups, but the SD could be decreased during the intervention and the groups became more homogenous.

To the best of our knowledge there are no data about the impact of triglycerides on DNA damage available.

4.2.8 The association between triglycerides and DNA damage in IDDM-T2 subjects

The IDDM-T2 subjects in the intervention group with low levels of triglycerides (≤ 1.2 mmol/l) displayed a reduction in the levels of oxidative DNA damage by 33.48% after 4 weeks and a significant decrease of 38.87% after 8 weeks when compared to baseline. A reduction in the amount of DNA damage was also seen in IDDM-T2 subjects with higher triglycerides (≥ 1.3 mmol/l) during the intervention after 4 weeks (-13.80%) and after 8 weeks (-21.67%). The decrease was insignificant due to the high SD at baseline (Fig. 12).

No significant changes were seen in levels of H_2O_2 resistance, FPG- and Endo III-sensitive sites during the intervention in IDDM-T2 subjects.

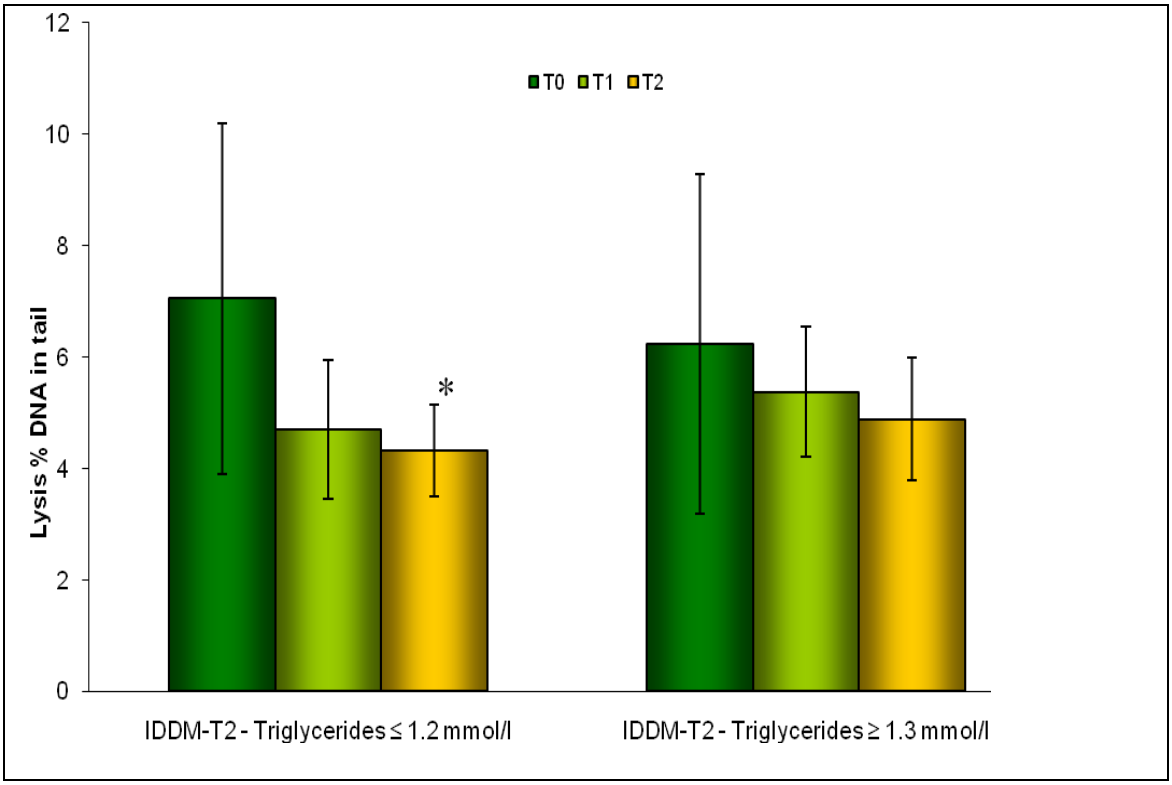


Fig. 12: DNA damage is significantly decreased in IDDM-T2 subjects with lower levels of triglycerides (≤ 1.2 mmol/l) in comparison to baseline after 8 weeks ($p \leq 0.05$; * indicates significant differences from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

A high reduction in oxidative DNA damage by nearly 40% was found in the IDDM-T2 subjects with lower triglyceride concentrations after 8 weeks of intervention. The subjects with higher amounts of triglyceride levels showed also a decrease by 22% at the end of the intervention period (T2).

In summary we showed that the daily consumption of vegetables and plant oil has in the group with IDDM-T2 subjects very positive effects on DNA damage when considered to the levels of triglyceride concentrations.

4.2.9 **The association of age and DNA damage**

Elderly subjects (aged ≥ 60 years) of the intervention group showed a decrease in the levels of DNA damage after 4 weeks (-16.93%) and after 8 weeks levels were significantly reduced by 19.96% when compared to baseline. In younger subjects (aged ≤ 59.9 years) decreases in DNA damage were seen also after 4 weeks (-20.12%) and after 8 weeks (-23.67%), but these results were statistically not significant. The main reason for the insignificant change in this group was the high SD at baseline (Fig. 13).

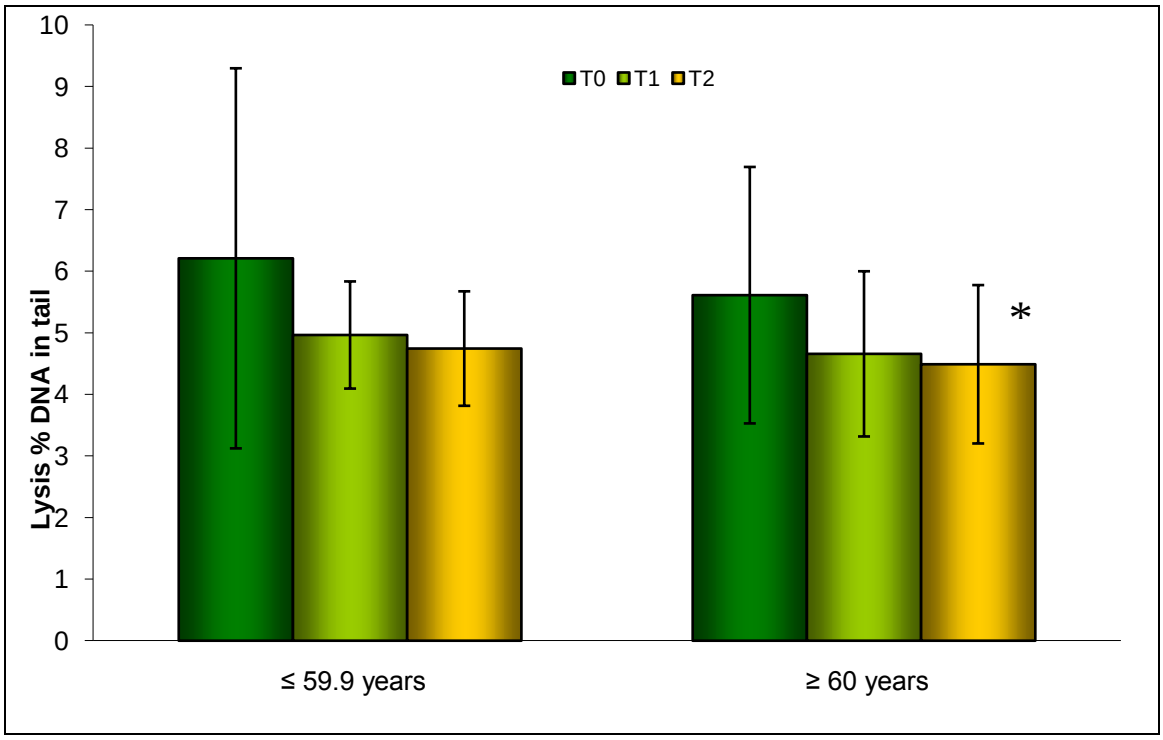


Fig. 13: Subjects aged ≥ 60 years showed significantly lower levels of DNA damage after the intervention in comparison to baseline (p ≤ 0.05; * indicates significant differences from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

In this study, younger subjects (≤ 59.9 years) showed a significant decrease in the amounts of Endo III-sensitive oxidative DNA damage after 4 weeks when compared to baseline (T0: 2.19 ± 2.14, T1: 1.26 ± 0.86). No significant changes were seen in the

resistance to H_2O_2-induced DNA damage and oxidised purines during the intervention period.

After the return to normal diet period at week 16, no significant effects on DNA damage in comparison to baseline and each intervention time point were seen in both age groups.

Zhao et al. reported similar findings in a study with older healthy woman (aged between 50 – 70 years). After supplementation with single carotenoids or mixed carotenoids for 56 days, the levels of endogenous DNA damage were decreased [ZHAO et al., 2006]. A long term supplementation with extracts of vegetables and fruits for 80 days in elderly subjects (mean age 68 years) showed a significant decrease in the amount of DNA damage [SMITH et al., 1999]. Giovannelli et al. found no association between oxidative DNA damage and age [GIOVANNELLI et al., 2002].

In conclusion the total study group showed positive effects on DNA damage during the intervention. In the group with the elderly subjects the reduction was significant with a decrease by 20%. Although the younger subjects showed a higher reduction in the levels of oxidative DNA damage with nearly 24%, these results were not significant. Responsible for that was the high SD at the beginning of the study (T0). The studies mentioned above showed also significantly reduced levels of DNA damage but after supplementation. A diet rich in vegetables and plant oil has the same beneficial effects on DNA damage.

4.2.10 **The association of age and DNA damage in IDDM-T2 subjects**

In our study, no significant changes in the intervention group with IDDM-T2 on DNA damage were found when divided in two age groups. The group with the younger IDDM-T2 patients (aged ≤ 59.9) showed a decrease by 35.47% in DNA damage after 4 weeks when compared to baseline. The levels of DNA damage moderately increased again in week 8, but the reduction in comparison to baseline was still high with 32.82% (T0: 7.19 ± 4.35, T1: 4.64 ± 0.19, T2: 4.83 ± 1.16). These results were not significant due to the high SD at baseline.

In elderly subjects with IDDM-T2 the levels of DNA damage were insignificantly lower after 4 weeks (-16.88%) and tended to decrease after 8 weeks (-24.84%) when compared to baseline ($p = 0.063$; T0: 6.40 ± 2.55, T1: 5.32 ± 1.40, T2: 4.81 ± 1.38). Worth mentioning in this context are two antioxidant supplementation studies that

investigated the association between DNA damage and age in subjects with diabetes. An intervention trial with 400 IU α-tocopherol supplements for 8 weeks showed, that age was not significantly related to DNA damage in subjects with T2DM [SAMPSON et al., 2001]. On the other hand, Lean et al. reported significantly decreased oxidative DNA damage in elderly T2DM subjects (age: 60.1 ± 7 years) after supplementation with dietary flavonols for 2 weeks [LEAN et al., 1999]. Our findings indicate that age is related to the levels of DNA damage in subjects with IDDM-T2. The younger IDDM-T2 subjects of our study showed a reduction of around 33% at the end of the intervention period (T2) in comparison to T0. In elderly subjects also a decrease in DNA damage with nearly 25% was seen after 8 weeks with respect to baseline. Although both age groups showed a high reduction, our results were not significant due to the high SD at baseline. In conclusion, IDDM-T2 subjects aged ≤ 60 years showed a better response to daily intake of vegetables and plant oil than the elderly subjects with IDDM-T2.

5 Conclusion

Our results demonstrate that daily intake of 300 g vegetables and 25 g of plant oil significantly decrease the levels of DNA damage. In subjects with IDDM-T2 the decrease was usually higher than in the total study group. That is because IDDM-T2 subjects have a better response to the dietary intervention than healthy individuals. The protective effects of AOs against DNA damage may be more evident in diabetics.

The parameters HbA1c, fasting plasma glucose, triglyceride levels and age had an impact on oxidative DNA damage. Reductions in the levels of DNA damage were seen when considering to these parameters.

At baseline the groups did not differ in the levels of oxidative DNA damage when compared with their stage of health. At the beginning of the study IDDM-T2 subjects with higher HbA1c (≥ 8.0%) showed significantly higher H_2O_2-induced DNA damage than IDDM-T2 subjects with lower HbA1c (≤ 7.9%). The levels of oxidised purines were significantly increased in subjects with high fasting plasma glucose (≥ 6.21 mmol/l) when compared to subjects with normal fasting glucose (≤ 6.2 mmol/l).

The total study group showed significantly lower levels of DNA damage after the intervention period when compared to baseline ($p \leq 0.05$). Subjects with HbA1c ≤ 7.9% displayed significantly lower levels of DNA damage ($p \leq 0.05$). No significant changes of oxidative DNA damage were observed in the total study group when considering their fasting plasma glucose and triglyceride levels. Elderly subjects aged ≥ 60 years showed significantly reduced DNA damage at the end of the intervention ($p \leq 0.05$).

Subjects with IDDM-T2 displayed significantly reduced DNA damage after 8 weeks in comparison to baseline ($p \leq 0.05$). HbA1c was also related to DNA damage in subjects with IDDM-T2 during the intervention. A significant decrease in DNA damage was seen ($p \leq 0.05$). A high but insignificant reduction of 30% in DNA damage was found in IDDM-T2 subjects with HbA1c ≥ 8.0%. Oxidative DNA damage was tended to decrease in IDDM-T2 subjects with higher concentration of fasting plasma glucose ($p \leq 0.15$). The DNA damage in IDDM-T2 subjects with lower triglyceride concentrations was significantly decreased with a reduction of 39% ($p \leq 0.05$). In elderly subjects with IDDM-T2 the levels of DNA damage tended to decrease after the intervention period ($p \leq 0.15$). The younger IDDM-T2 subjects showed no significant changes, but the reduction in DNA damage was very high with about 35%.

Further dietary intervention studies with antioxidants contained in vegetables and plant oils are needed to support the hypothesis of the beneficial effects on the levels of DNA damage in T2DM subjects.

6 Summary

T2DM and its late complications are associated with higher oxidative stress, and may lead to increased DNA damage. Vegetables and plant oils, which are good sources of antioxidants have been suggested to reduce oxidative stress and the levels of DNA damage.

In a randomised intervention trial at the Department of Nutritional Sciences in the Emerging Field "Oxidative Stress and DNA Stability" in Vienna, we studied 76 subjects with diabetes (41 NIDDM and 35 IDDM-T2), 12 subjects with IFG and 11 healthy individuals. All subjects were randomly assigned to control or intervention group. The control group received information about a healthy diet. The intervention group was requested to consume 300 g of vegetables and 25 g of a plant oil per day together with their usual diet for 8 weeks. Lymphocyte DNA damage was measured at baseline, after 4 weeks, after 8 weeks (end of intervention period) and after 16 weeks. DNA damage, resistance to H_2O_2-induced DNA damage and oxidised bases were evaluated by comet assay. Several parameters, which may have an impact on DNA damage were measured such as HbA1c, fasting plasma glucose and triglycerides. The association of age and DNA damage was also evaluated.

The intervention led to a significant reduction of DNA damage in the total study group and IDDM-T2 subjects after 8 weeks in comparison to baseline ($p \leq 0.05$). A slight but not significant increase in the resistance to H_2O_2-induced DNA damage was also found in the total study group. Levels of oxidised bases did not change significantly during the intervention. In the control group there were no changes in any marker of oxidative DNA damage. IDDM-T2 subjects with HbA1c $\leq$ 7.9% had significantly decreased levels of DNA damage after the intervention period in comparison to baseline ($p \leq 0.05$). The IDDM-T2 subjects with triglyceride levels $\leq$ 1.2 mmol/l showed significantly reduced DNA damage when compared to baseline ($p \leq 0.05$).

In summary, we demonstrated that our intervention with a daily consumption of vegetables and a plant oil decreases oxidative DNA damage in subjects with diabetes.

7 Zusammenfassung

Diabetes mellitus Typ 2 (DM-II) und die diabetischen Folgeerkrankungen stehen im Zusammenhang mit oxidativen Stress und können zu erhöhten DNA-Schäden führen. Es wird angenommen, dass Gemüse und Pflanzenöle durch ihren hohen Gehalt an Antioxidantien, oxidativen Stress und dadurch das Ausmaß an DNA-Schäden reduzieren können.

Eine randomisierte Interventionsstudie wurde am Institut für Ernährungswissenschaften in der Arbeitsgruppe „Oxidative Stress and DNA Stability“ in Wien durchgeführt. Es wurden 76 Probanden mit DM-II (41 nicht-insulinabhängige und 35 insulinabhängige DM-II), 12 Probanden mit gestörter Nüchternblutglukose sowie 11 Gesunde untersucht, wobei alle Probanden zufällig der Kontroll- oder Interventionsgruppe zugeteilt wurden. Die Kontrollgruppe erhielt Informationen über eine gesunde Ernährungsweise. Die Interventionsgruppe wurde aufgefordert täglich 300 g Gemüse und 25 g eines Pflanzenöls für 8 Wochen ohne zusätzliche Veränderungen des Ernährungsverhaltens zu konsumieren. Die DNA-Schäden in den isolierten Lymphozyten wurden zu Beginn der Studie, nach 4 und nach 8 Wochen der Intervention (Beendigung der Interventionsphase), sowie nach 16 Wochen gemessen. Die DNA-Schäden, Resistenz gegenüber Wasserstoffperoxid induzierten DNA-Schädigung und oxidierte Basen wurden mittels Comet Assay untersucht. Verschiedene Parameter wie HbA1c, Nüchternblutglukose und Triglyceride wurden in die Untersuchungen miteinbezogen, da sie einen Einfluss auf DNA-Schäden haben könnten. Der Zusammenhang zwischen Alter und DNA-Schäden wurde ebenfalls untersucht.

Die Intervention zeigte eine signifikante Reduktion an DNA-Schäden im Gesamtkollektiv und in der Gruppe der insulinpflichtigen DM-II Probanden nach 8 Wochen im Vergleich zu Beginn der Studie ($p \leq 0.05$). Eine leichte, aber nicht signifikante Steigerung der Resistenz durch Wasserstoffperoxid verursachten DNA-Schädigung konnte im Gesamtkollektiv gemessen werden. Der Gehalt an oxidierten Basen änderte sich während der Intervention nicht. Die Kontrollgruppe zeigte keine signifikanten Veränderungen der DNA-Schäden bei den verschieden Parametern. Insulinpflichtige DM-II Probanden mit einem HbA1c von $\leq$ 7.9% wiesen eine signifikante Abnahme der DNA-Schäden nach der Intervention im Vergleich zu Beginn der Studie auf ($p \leq 0.05$).

Ebenfalls waren bei den insulinpflichtigen DM-II Probanden mit einer Triglyceridkonzentration von ≤ 1.2 mmol/l die DNA-Schäden im Vergleich zu Beginn der Studie signifikant reduziert ($p \leq 0.05$).

Zusammenfassend konnten wir zeigen, dass eine Intervention mit täglichem Gemüse- und Pflanzenölkonsum den Gehalt an oxidierten DNA-Schäden bei Diabetikern senken kann.

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Education

02/2003 – 10/2004	Vocational matriculation examination
Since 10/2004	Study of Nutritional Sciences at the University of Vienna

Working Experience

July 2008	Intership at the University of Vienna, Department of Nutritional Sciences
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