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Influence of a Dietary Intervention on oxidative DNA Damage in Non-insulin-dependent Diabetes Mellitus Type 2

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Meiner lieben Familie

Es ist nicht genug zu wissen - man muss auch anwenden. Es ist nicht genug zu wollen - man muss auch tun.

Johann Wolfgang von Goethe

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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 and Hypothesis

According to the International Diabetes Federation approximately 285 million people worldwide suffered from Diabetes mellitus in the year 2010. According to the "Austrian diabetes report" 600 000 diabetics in Austria were estimated in the same year. Approximately 95% of people suffering from diabetes have Diabetes mellitus Type 2 (T2DM), which it is formally known as Non-insulin-diabetes mellitus. T2DM is now one of the most common diseases worldwide and its prevalence is expected to increase by 7.7 % in 2030.

DMT2 is a heterogenous disorder characterized by impaired insulin secretion and insulin action that leads to hyperglycaemia. Elevated free fatty acids and oxidative stress exacerbate the metabolic abnormalities in diabetes and its long-term complications, and may be involved in the pathogenesis of this disease. Oxidative Stress may also lead to high levels of DNA damage, and it has also been linked to insulin resistance and beta cell dysfunction, which may cause increased damage to deoxyribonucleic acid (DNA).

The hypothesis that a daily consumption of vegetables rich in antioxidants and vitamins in combination with a plant oil rich in polyunsaturated fatty acids (PUFAs) may have protective effects on oxidative DNA damage and the development of late complications in diabetics, provided the basis to perform the DIAPLANT study. The aim of this experimental study was to evaluate the influence of a daily intervention of 300 g vegetables and 25 ml plant oil for eight weeks on oxidative DNA damage in T2DM. This thesis is a part of the DIAPLANT study and investigated especially the effect of this intervention on subjects with non-insulin-dependent T2DM.

The thesis was written together with Simone Pleifer. We performed the experimental part of this thesis study and the chapters "Literature survey" and "Material and Methods" were written together and are identical.

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 hyperglycaemia. Hyperglycaemia 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 Tab. 1.

The status of pre diabetes occurs when abnormalities of glucose metabolism exist but the diagnostic criteria for diabetes are not met. Tab. 2 shows the diagnostic criteria.

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)
Haemoglobin 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 into various tissues. Depending on the blood 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 which 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 hyperglycaemia. 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 oedema, 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 properly diabetes can impair nerves. The overload of sugar can injure the capillary walls 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, diarrhoea 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 increased 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, hyperlipidaemia, insulin resistance and obesity [JÖNSSON, 2002]. In addition diabetes may lead to lower bone mineral density and increased 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 and 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 from 1997 to 2007 in order 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 worldwide [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

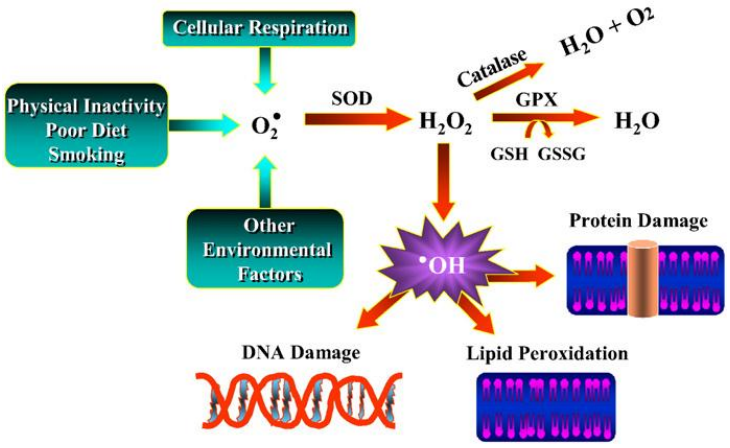
of diabetes are required for policy decisions, in order to upgrade the allocation of resources and to evaluate the success of different attempts for disease management. The "CODE-2 study" described the first coordinated effort to measure the cost caused by people with T2DM in Europe. This study calculated 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 2.834,-.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 cigarettes, 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]. This is shown in 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. Fig. 2 shows that Oxidative stress results from an increased level of ROS and/or decreased antioxidant defence. 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].

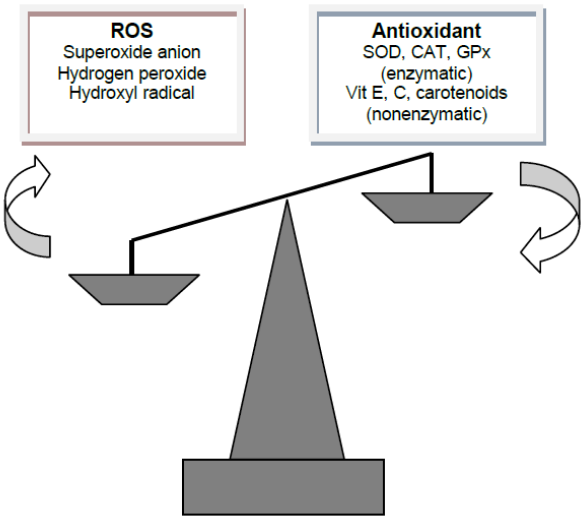


Fig. 2: Imbalance between pro-oxidants and antioxidants

(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. Hyperglycaemia 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 hyperglycaemia [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 animal 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].

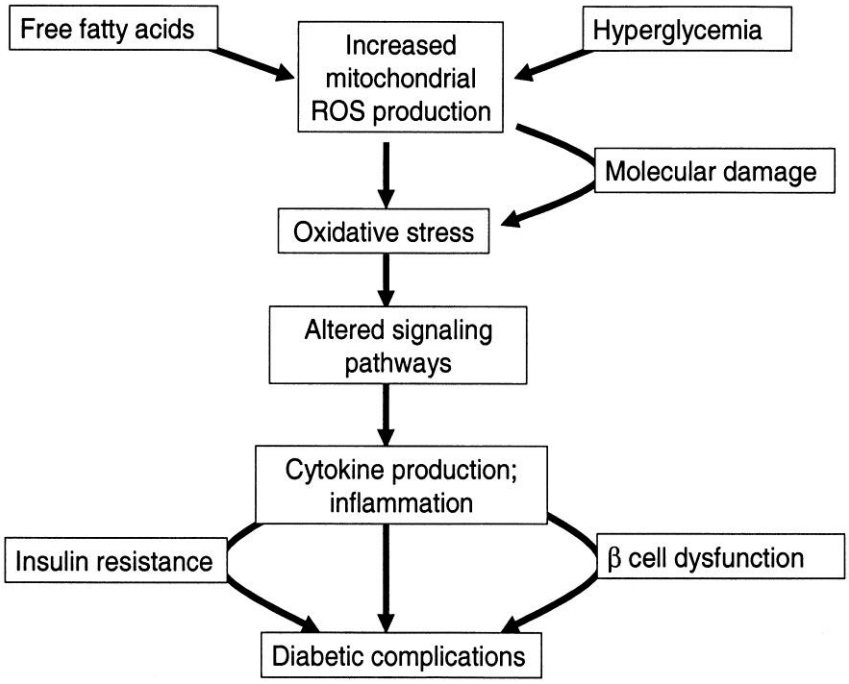


Fig. 3: Overview of the development of diabetic complications

(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]. The mechanism of enzymatic antioxidants is shown in 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 **Non-enzymatic antioxidants**

The most common non-enzymatic 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 mainly 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 is β-carotene and lycopene. Polyphenols are radical scavengers and can inhibit the peroxidation of lipids and some 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, also known as single cell gel electrophoresis assay (SCGE) has become one of the standard methods for assessing oxidative DNA-damage. It is a method for measuring DNA strand breaks in eukaryotic cells. Lymphocytes are often used in human studies, since they are easy to obtain. Furthermore they circulate through the whole body and therefore reflect the level of damage in the whole organism [COLLINS et al., 2008]. Ostling and Johanson configured the Comet assay for the detection of double strand breaks in mammalian cells for the first time in the year 1984 [OSTLING and JOHANSON, 1984]. The method was further optimized by Singh et al. [SINGH et al., 1988] and Olive et al. [OLIVE et al., 1991]. Under alkaline conditions the SCGE assay allows the detection of single-strand breaks, double strand breaks, oxidised bases and one can measure the cells' sensitivity against external damage, as well as DNA repair. Lesion specific enzymes are able to convert oxidized pyrimidines and purines into additional DNA strand breaks. Endonuclease III (Endo III) recognizes oxidized pyrimidines while oxidized purines are recognized by Formamidopyrimidine glycosylase (FPG). Several dietary intervention trials, clinical and biomonitoring studies report on the use of SCGE assay for the detection of DNA damage [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 better 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 haemodialysis 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 non-smoking 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 non-smokers. After 20 weeks of supplementation a highly significant decrease in oxidative base damage could be shown in both smokers and non-smokers. 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 nine 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 nine 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 defines 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 non-smoking 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 led 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 no significant effects on DNA strand breaks and 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 samples 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 effects of omega 3 fatty acids, which have antiinflammatory, antithrombotic, antiarrhythmic, hypolipidaemic 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 results 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 non-smoking 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 non-smokers. The daily almond intake reduced DNA strand breaks by 34% in smokers compared with pre-treatment 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 supplementary 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. The study design is shown in 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). Tab. 5 shows the intervention design.

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: 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)			

3.1.2 **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 anti-diabetic drugs. Insulin and partly anti-diabetic 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. A description of the study collective is shown in 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

3.1.3 **Inclusion and exclusion criteria**

The inclusion and exclusion criteria for the participants of the study are listed in the Tab. 7 and Tab. 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*

3.1.4 Vegetables and plant oil

For the study we used a walnut-oil (intervention oil), which was rich in PUFAs and α-tocopherols. A list of the ingredients of this plant oil is shown in Tab. 9.

The frozen vegetables were kindly donated by “Iglo Austria”. The main focus was on green vegetables, which are rich sources of folic acid. Tab. 10 lists the vegetables available for the subjects during the intervention.

Tab. 9: Intervention oil

% of total fatty acids	
C16:0	7.28 ± 0.09
C18:0	2.06 ± 0.07
C18:1n9c	16.40 ± 0.99
C18:1n7c	0.95 ± 0.03
C18:2n6c	61.76 ± 1.00
C18:3n3	11.49 ± 0.43
mg/100 g oil	
γ-tocopherol	32.98 ± 1.82
α-Tocophero	2.76 ± 0.14

Tab. 10: 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 the study team.

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, nucleotides are incubated with lesion-specific repair enzymes. These enzymes convert oxidised bases to strand breaks. Endo III allow to detect oxidised pyrimidines and FPG recognises oxidised purines [COLLINS, 2004].

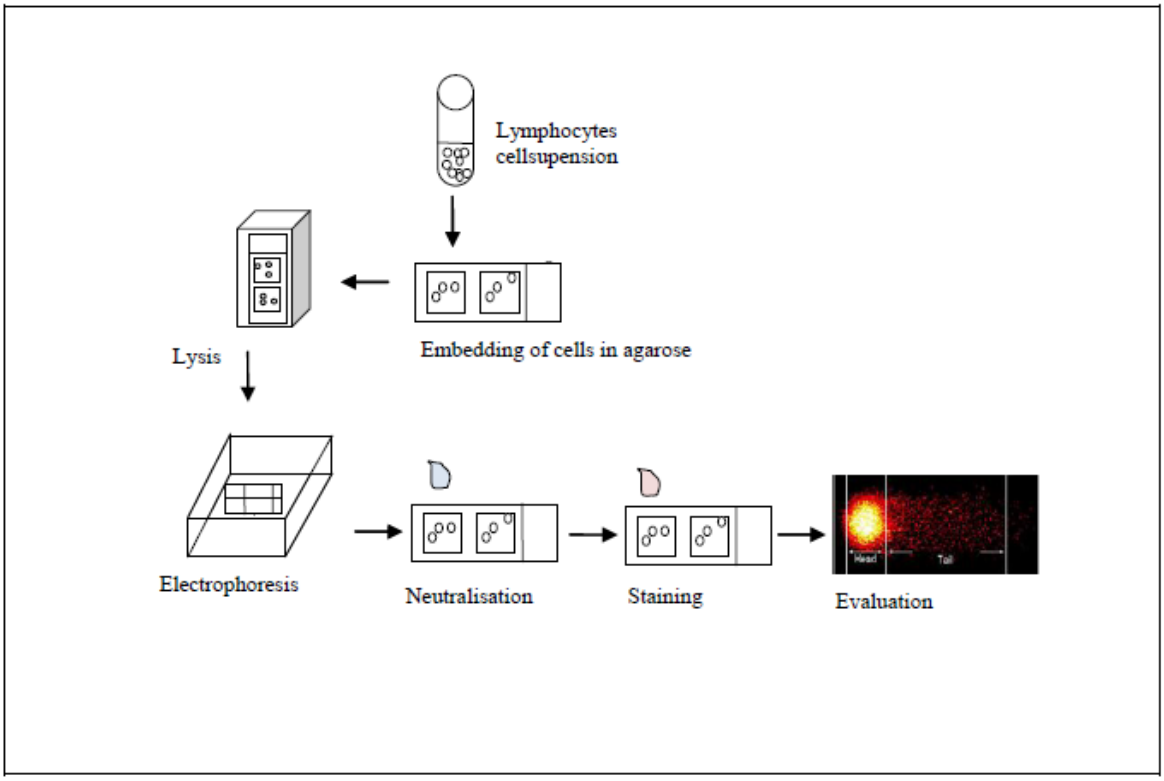


Fig. 4: Scheme of the comet assay

3.2.1 **Chemicals and working equipment**

3.2.1.1 **Chemicals and Reagents**

Tab. 11 shows a list with the used chemicals and reagents for the measurements with Comet assay.

Tab. 11: 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*	06203
	Sigma	
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. 12 shows the used equipment for the measurement for Comet assay.

Tab. 12: 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*
Watherbath (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 reaction 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 the night.

The pre-coated 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. Then it was centrifugated at 3100 rpm (rounds per minute) for 25 min at 22°C. 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 pre-coated with NMA were used and labelled with the subject-number and the kind of treatment: “lysis”, “H_2O_2”, “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 "H_2O_2" 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 the 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 clinical characteristics of the groups (control vs. intervention) at baseline are shown in Tab. 13. Body mass index (BMI), diabetes duration, HbA1c, triglycerides, fasting plasma glucose and C-peptide did not differ between both groups. Subjects of the control group were slightly but significantly younger than subjects of the intervention group.

Tab. 13: 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
C-peptide	0.75 ± 0.41	0.90 ± 0.56
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

Tab. 14 shows levels of DNA damage, oxidised bases (purines and pyrimidines) and resistance to H_2O_2-induced DNA damage of IDDM-T2DM, NIDDM, IFG- and healthy participants at baseline. There were no significant differences between the groups with different health status. Healthy subjects showed slightly, but not significantly higher levels of DNA damage than participants with T2DM. Also levels of oxidised purines and pyrimidines were not significantly greater in T2DM subjects when compared to the healthy participants.

Between the control and the intervention group only IDDM-T2 participants showed significantly higher levels of DNA damage (Lysis) at baseline. There were no significant differences between control and intervention group in the NIDDM-, IFG- and healthy in any marker of oxidative DNA damage (Lysis, FPG, Endo III and H_2O_2).

Tab. 14: Oxidative DNA damage in control and intervention group 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

Various studies indicate significantly higher levels of oxidative DNA damage in diabetics, when compared to healthy subjects [COLLINS et al., 1998; LODOVICI et al.,

2008; SONG et al., 2007]. Other studies describe significantly higher levels of FPG-sensitive sites in T2DM subjects when compared to healthy individuals [DINÇER et al., 2002; PITOZZI et al., 2003].

The results of this study do not show higher levels of DNA damage in subjects with diabetes. Our measurements are in accordance with results from Hannon-Fletcher et al. [HANNON-FLETCHER et al., 2000] and Ibarra-Costilla et al. [IBARRA-COSTILLA et al., 2010]. They suggest that there are no significant differences in levels of DNA damage between healthy and diabetic subjects. A possible explanation for not finding any differences within our study might be that the participants with diabetes were metabolically stable and displayed good glycaemic control.

4.1.3 Association between Connecting-peptide (C-peptide) and resistance to H_2O_2-induced DNA damage at baseline

T2DM is characterised by insulin resistance and relative insulin deficiency. Insulin is produced by beta-cells of the pancreas. First, pro-insulin is secreted, which can be split in C-peptide and insulin. Insulin and C-peptide are always produced in similar amounts and therefore C-peptide levels can be used as a marker of insulin secretion. Measurements of an elevated insulin level without increased C-peptide concentration indicate insulin treatment [HORN et al., 2002]. The participants of the whole study group (including healthy and diabetic subjects) displayed a weak none-significant association between C-peptide and resistance to H_2O_2-induced DNA damage ($r = -0.187$, $p = 0.073$) at baseline (T0). In NIDDM subjects this association was much stronger and significant ($r = -0.329$, $p = 0.047$). The correlation is shown in Fig.5. This result indicates that the more insulin is produced by the pancreas or the fewer beta-cells are destroyed, the smaller are the levels of DNA damage after treatment with 100 µM H_2O_2.

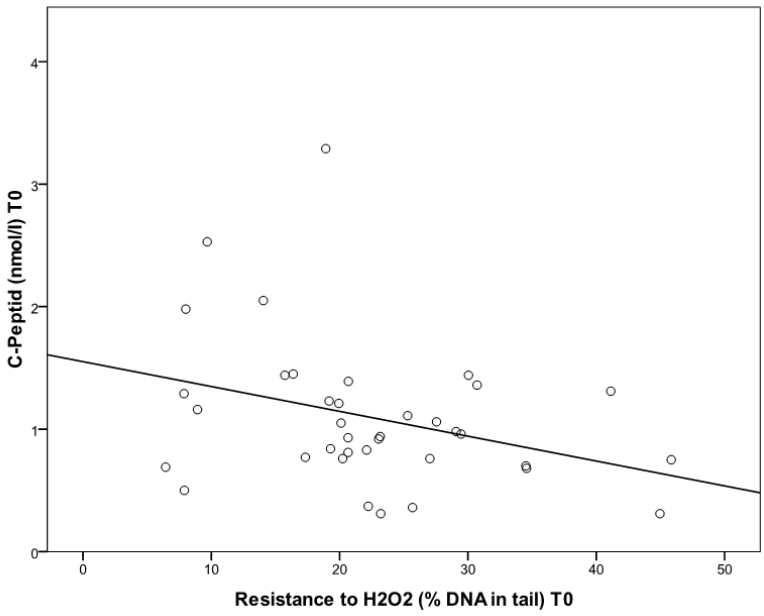


Fig. 5: Association between C-peptide and resistance to H_2O_2-induced DNA damage in subjects with NIDDM at baseline (r = -0.329, p = 0.047)

The aim of an animal experiment in Wistar rats was to investigate the association between oxidative stress and decreased beta-cell function induced by elevation of plasma free fatty acids (oleate infusion). The study group demonstrated that the permanent exposure to free fatty acids leads to decreased insulin and C-peptide response in beta-cells. Therefore they concluded that oxidative stress has impact on beta-cell secretion [OPRESCU et al., 2007]. If we combine the results of Orpescu et al. with findings in our study one could conclude that increased oxidative stress leads to decreased beta-cell function, which is associated with reduced resistance of lymphocytes to H_2O_2.

4.1.4 Impact of glycaemic control (HbA1c and fasting plasma glucose) on oxidative DNA damage

In order to evaluate the influence of glycaemic control on DNA damage the whole study group was subdivided into good (HbA1c $\leq$ 7%) and poor (HbA1c > 7%) glycaemic controlled subjects. The measurements showed no significant differences in oxidised DNA damage and also not in resistance to H_2O_2-induced DNA damage at baseline.

Dinçer et al. evaluated in T2DM subjects with poor glycaemic control significantly higher levels of FPG-sensitive oxidative DNA damage. In comparison to good controlled subjects 63 T2DM subjects and 41 healthy controls participated in this study.

Subjects were subdivided into well controlled and poor controlled (> 6.2%) diabetics according to their HbA1c levels [DINÇER et al., 2002]. Lodovici et al. demonstrated in 39 T2DM subjects that poor glycaemic controlled T2DM subjects had a slightly lower plasma antioxidant status and greater levels of DNA damage when compared to healthy controls (n = 18). In their study the subjects with good glycaemic control were defined as HbA1c ≤ 7% and with serum glucose concentration ≤ 139 mg/dl (7.7 mmol/l) [LODOVICI et al., 2008].

When our total study group was subdivided into the group according to their fasting plasma glucose, the subjects with fasting plasma glucose levels > 6.2 mmol/l showed significantly higher levels of oxidised purines than the subgroup of subjects with normal fasting plasma glucose (≤ 6.2 mmol/). These effects are shown in Fig.6.

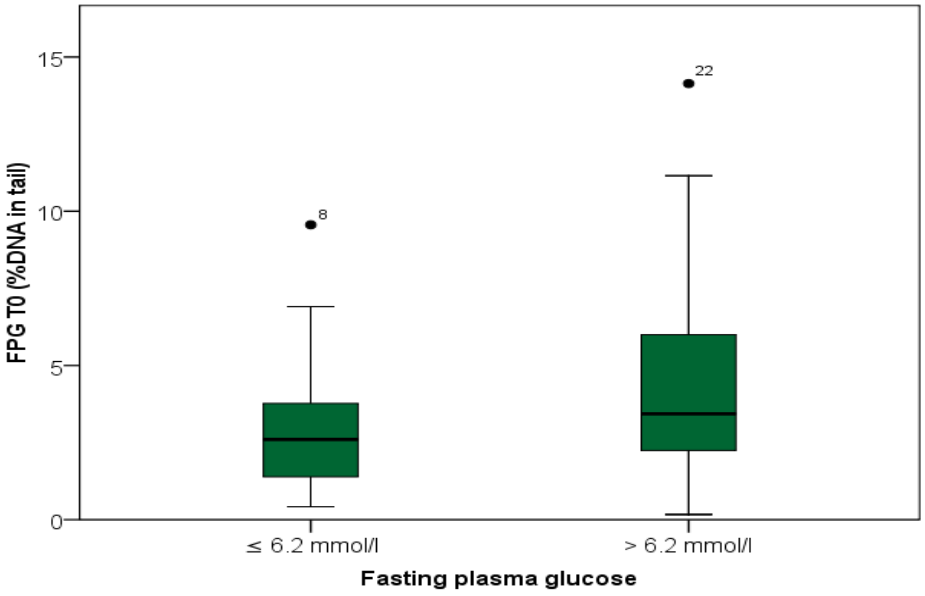


Fig. 6: Oxidised purines in subjects with normal and elevated levels of fasting plasma glucose at baseline (p ≤ 0.05).

Our findings support the hypothesis that hyperglycaemia results in increased oxidative stress leading to elevated oxidative DNA damage.

NIDDM subjects with high fasting plasma glucose levels did not show significantly higher levels of FPG-sensitive DNA lesions (3.99 ± 2.62) compared to subjects with low fasting plasma glucose (2.41 ± 1.52).

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

an association between higher FPG-sensitive DNA damage and higher concentration of plasma glucose in subjects with T2DM [LODOVICI et al., 2008]. Choi et al. found that DNA-damage is significantly correlated with fasting plasma glucose and HbA1c. Furthermore they reported that oxidative DNA damage was significantly higher in T2DM subjects with poor glycaemic control and low levels of plasma ascorbic acid (< 47.7 μM) than in subjects with a similar degree of hyperglycaemia but higher concentrations of plasma ascorbic acid (≥ 47.7 μM) [CHOI et al., 2005].

4.1.5 **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.

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].

To the best of our knowledge there are no data so far published about an association between BMI or diabetes duration and oxidative DNA damage in T2DM.

4.2 Effect of the intervention

The following chapters which report the effects in the whole study group, the control and the intervention group, were written together with Simone Pleifer and are identical with the thesis "Influence of a Dietary Intervention on oxidative DNA-damage in Insulin-dependent Diabetes mellitus Type 2".

Furthermore the following chapters describe the effect of the intervention on subjects with NIDDM in comparison to NIDDM controls. The measured parameters HbA1c, fasting plasma glucose, triglycerides and age showed an influence on oxidative DNA damage in these participants. An aim of this study was also to verify the association of the intake of mixed vegetables and plant oil which led to increased plasma vitamin concentrations and levels of lymphocyte DNA damage. 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 by 20.77% in DNA damage was seen when compared to baseline. This is shown in Fig. 7.

The intervention group also displayed positive effects in the resistance to H_2O_2-induced DNA damage, but the differences were not significant (T0: 23.32 ± 10.59, T1: 23.11 ± 7.88, T2: 21.76 ± 7.45, T3: 22.86 ± 7.99). No effects were seen in the levels of FPG- and Endo III-sensitive oxidative DNA damage.

The results showed slightly higher levels of oxidative DNA damage 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.

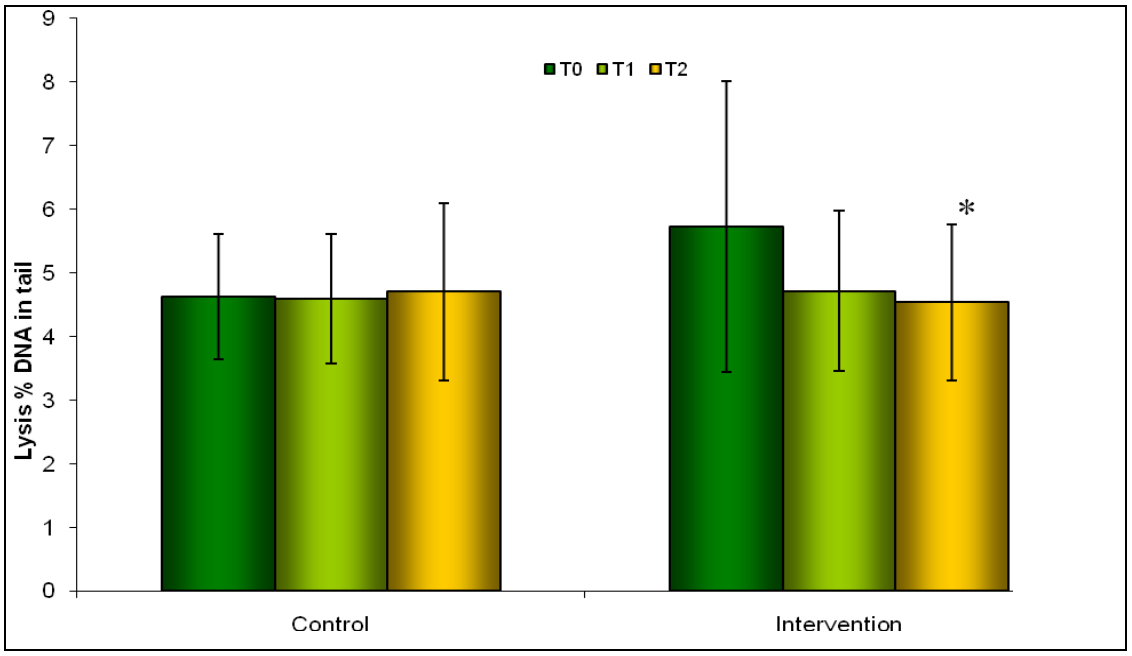


Fig. 7: Levels of DNA damage were significantly decreased in the intervention group but remained constant in the control group ($p \leq 0.05$; * indicates a significant difference from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

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 on 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 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 reduced DNA damage was significant. 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 a concentration up to 500 µmol/l.

4.2.2 **Effect of the intervention on NIDDM**

NIDDM subjects in the intervention-group showed slightly, but not significantly higher levels of oxidative DNA damage than the volunteers in the control-group at baseline (Tab.12).

During the intervention-period the volunteers showed significant effects after 4 weeks (a reduction by 16.57%, T1). The changes were also significant after 8 weeks (T2) of intervention with a reduction by 18.29% in respect to baseline (T0). These effects are shown in Fig. 8 (control group: T0: 4.36 ± 0.87, T1: 4.36 ± 0.77, T2: 4.80 ± 1.36 and T3: 4.74 ± 1.25; intervention group: T0: 5.25 ± 1.47, T1: 4.38 ± 1.07, T2: 4.29 ± 1.03 and T3: 4.54 ± 0.97).

The intervention and control group of NIDDM subjects represented no significant changes after 16 weeks when the subjects returned to usual diet (T3) in comparison to T0.

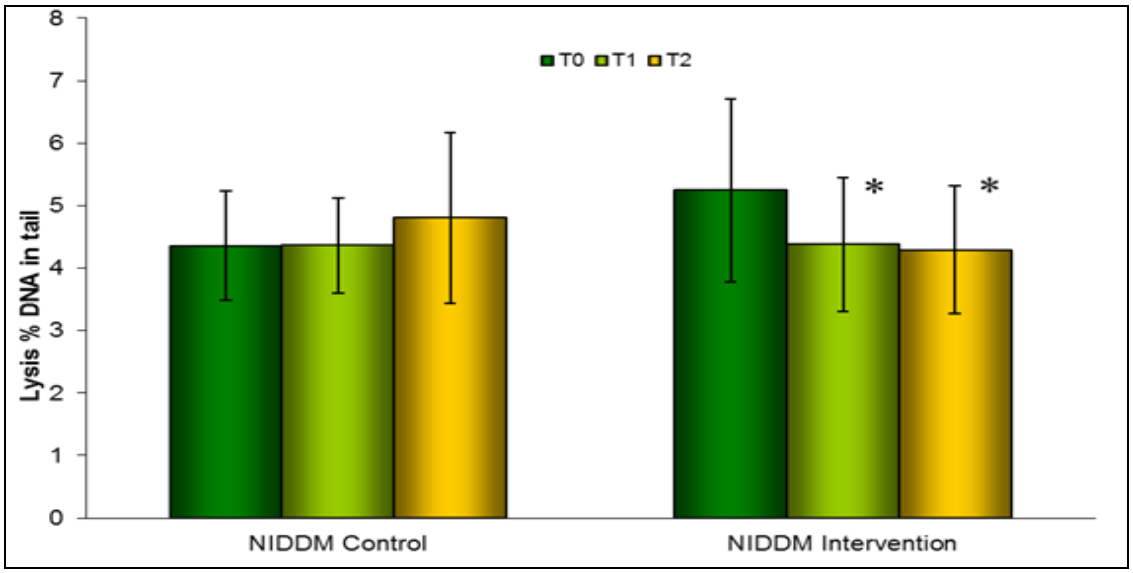


Fig. 8: The NIDDM subjects showed significantly decreased levels of oxidised DNA damage after 4 and 8 weeks of the intervention period, while levels remained constant in control group ($p < 0,05$; * indicates significant differences from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

The NIDDM subjects showed no significant changes in H_2O_2-induced oxidative DNA damage during the 8 weeks of intervention and after return to usual diet (T0: 23.50 ± 11. 22, T1: 22.30 ± 8.08, T2: 21.24 ± 8.45 and T3: 21.35 ± 7.08). The high SD was mainly responsible for the insignificant changes.

The level of oxidised purines decreased insignificantly by 34.43% after 4 weeks of intervention (T1), but during the following 4 weeks the levels of FPG-sensitive sites increased (T0: 3.34 ± 2.57, T1: 2.19 ± 1.44, T2: 3.18 ± 2.12 and T3: 3.48 ± 1.78). The intervention in NIDDM subjects also showed effects on oxidised pyrimidines. There was a reduction by 38.28% detected after 4 weeks of intervention (T1), but the level of ENDO III-sensitive sites increased after 8 weeks of intervention (T2) the level of ENDO III-sensitive sites increased (T0: 1.28 ± 1.03, T1: 0.79 ± 0.72, T2: 1.53 ± 1.40 and T3: 2.12 ± 1.67). We also measured significantly higher levels of ENDO III-sensitive sites after 8 weeks in the control group of NIDDM subjects (T0: 0.79 ± 0.56, T1: 1.72 ± 1.64, T2: 2.20 ± 0.22 and T3: 2.49 ± 1.59). The whole study period lasted 10 months and blood was collected in different seasons of the year. It may be that the variety of seasonal changes influenced the amount of oxidised DNA damage in the subgroup of NIDDM subjects. Smolková et al. studied nutritional parameters and markers of oxidative stress in three Slovak population groups to look for an association of seasonal variation in diet. The consumption of vegetables was in summer and autumn twice as high as in winter and spring. They also observed that the DNA damage did not vary consistently across the seasons [SMOLKOVÁ et al., 2004].

Astley et al. and Sampson et al. 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[ASTLEY et al., 1999, SAMPSON et al., 2001]. On the other hand, a daily supplementation with high amounts of α–tocopherol (900 IU) for 12 weeks showed significantly decreased levels of DNA damage in patients with T2DM and T1DM [ŞARDAŞ et al., 2001]. These results indicate that only high dose supplements of α-tocopherol over a long time period could have beneficial effects on oxidative DNA damage in diabetics.

After 2 weeks of a flavonol-rich diet (supplementation 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 levels of Endo III-sensitive oxidative DNA damage [LEAN et al., 1999]. These results indicate that also a short intervention with flavonol supplements has protective effects on DNA damage in subjects with T2DM.

In summary we could demonstrate that an intervention with natural AOs from vegetables and plant oil reduces the amount of DNA damage in subjects with T2DM. In comparison to the mentioned supplementation studies the results of our study indicate that dietary intake of a variety of different vegetables and plant oil have more beneficial influence on DNA damage than supplementation trials. Therefore it is possible to decrease DNA damage and probably also late diabetic complications by improving the diet.

4.2.3 The impact of HbA1c on DNA damage in the intervention group

Poorly controlled subjects (HbA1c > 7%) had slightly, but not significant higher basal levels of DNA damage in comparison to the better controlled subjects (HbA1c ≤ 7%) of the intervention group. Even after 4 weeks of consumption the oxidised DNA damage decreased significantly by 23.79% in the subjects with HbA1c levels > 7%. Levels decreased by 25.5% after 8 weeks of intervention. In contrast the better controlled subjects showed only a small decrease (- 10.4%) after 4 weeks. They showed significant reduced levels of DNA damage (- 15.18%) after 8 weeks. The levels of oxidative DNA damage in subjects with poor and better glycaemic control were not different any longer after 8 weeks of intervention. These effects are shown in Fig. 9.

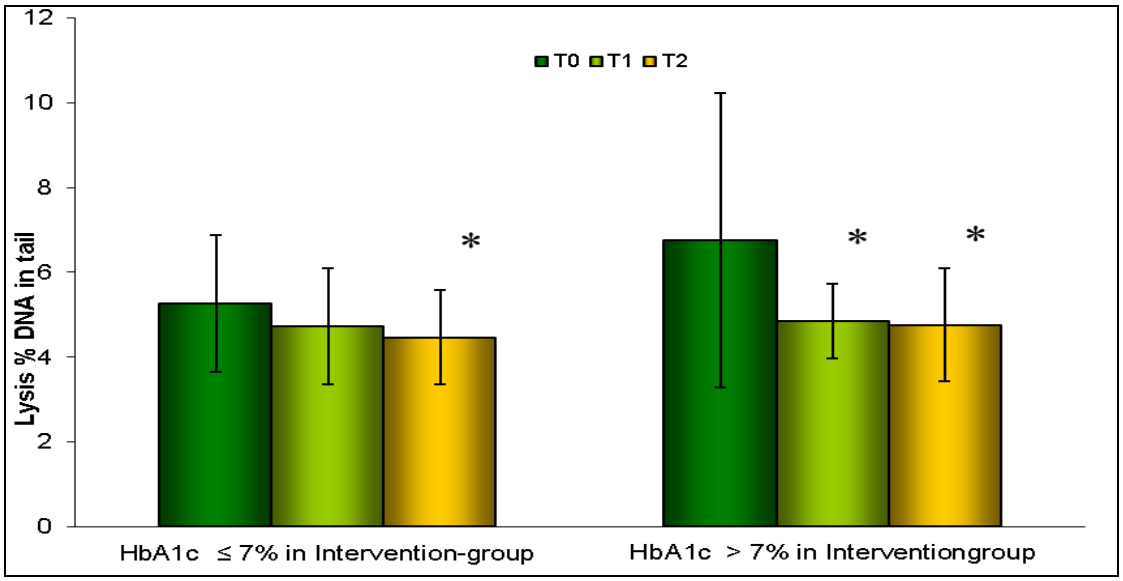


Fig. 9: Subjects with good glycaemic control (HbA1c ≤ 7%) and poor glycaemic control (HbA1c > 7%) showed significantly decreased levels of oxidised DNA damage during and after the intervention-period of 8 weeks (p ≤ 0.05; * indicates a significant difference from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

The effects of the intervention on resistance to H_2O_2-induced DNA damage are shown in Fig. 10. We detected a reduction by 14.24% in oxidative DNA damage after treatment with H_2O_2 in subjects with -poor glycaemic control (T0: 25.07 ± 10.86, T1: 23.03 ± 6.88 and T2: 21.51 ± 6.43). The better controlled volunteers showed no changes in resistance to H_2O_2-induced DNA damage (T0: 21.57 ± 10.21, T1: 23.19 ± 8.87 and T2: 22.02 ± 8.44).

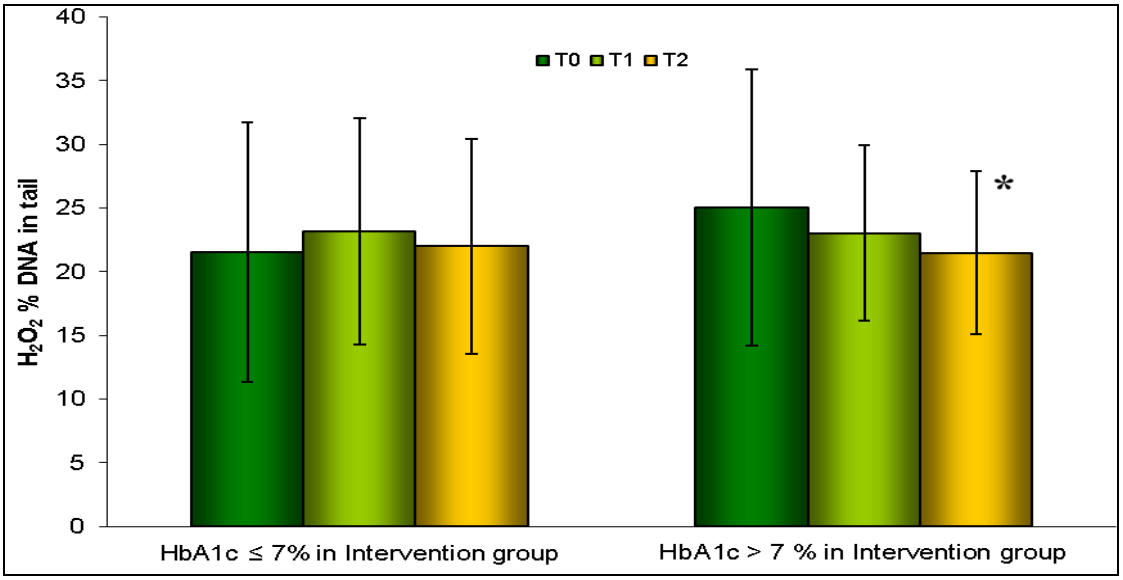


Fig. 10: Subjects with poor glycaemic control (HbA1c ≤ 7%) showed a significant decrease in H_2O_2-induced DNA damage after 8 weeks after of the intervention ($p \leq 0.05$; * indicates a significant difference from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

Further statistical analysis confirm with the association between HbA1c and the amount of oxidative DNA damage (Lysis, oxidised purines and resistance to H_2O_2 –induced DNA damage). The results display significant association between the changes in HbA1c (T2 – T0 = Δ) and the change in oxidative DNA damage during the intervention. This indicates that an improvement in glucose metabolism leads to decreased levels of DNA damage. These effects are shown in Fig. 11, Fig. 12 and Fig. 13. There was no correlation seen between △ oxidised pyrimidines △ HbA1c in the intervention group. None of the associations could be observed in the control group.

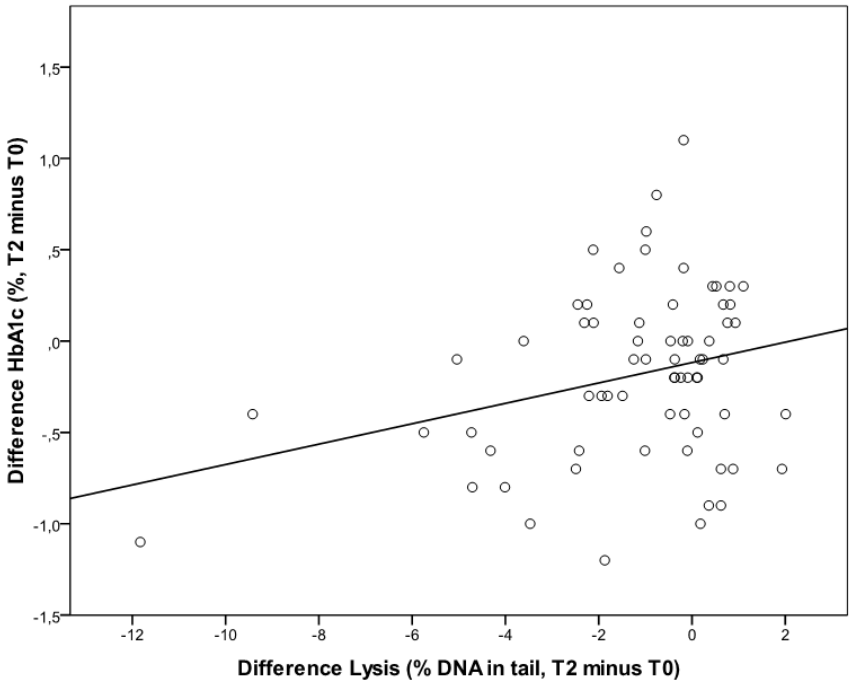


Fig. 11: Association between △HbA1c and △ Lysis in the intervention group (r = 0.285, p = 0.018).

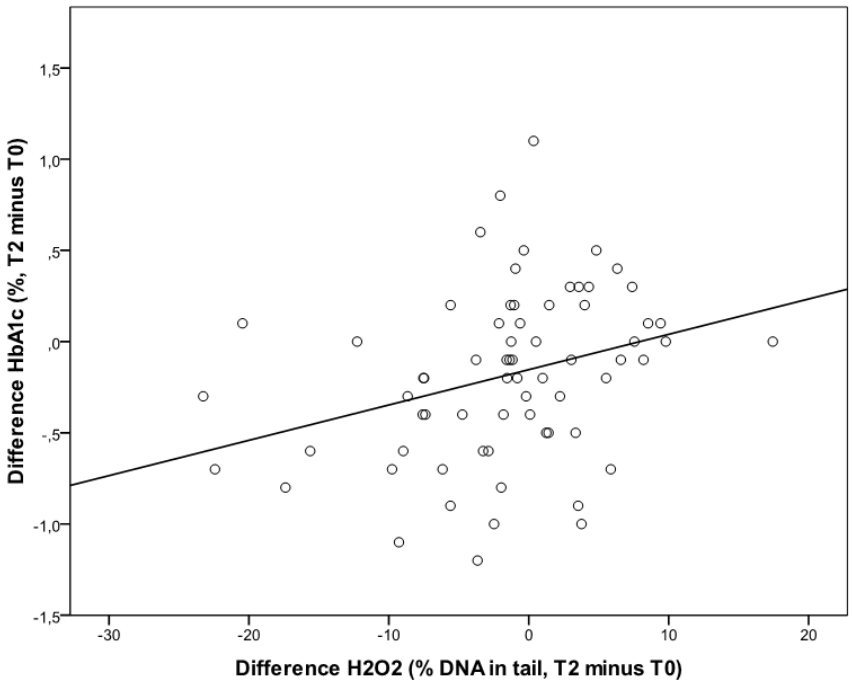


Fig. 12: Association between △ HbA1c and △ resistance to H_2O_2-induced DNA damage in the intervention group (r = 0.313, p = 0.009).

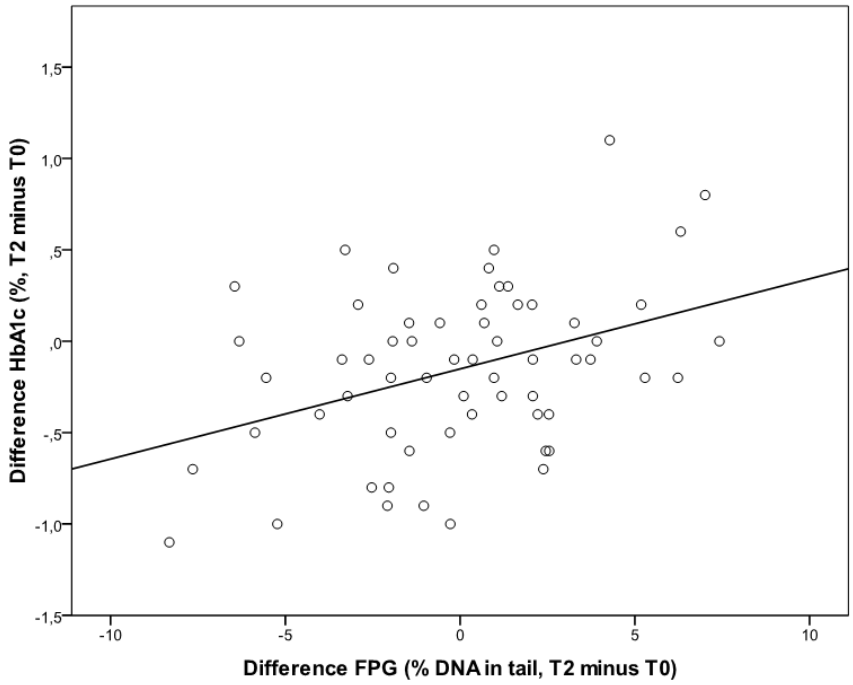


Fig. 13: Association between △ HbA1c and △ oxidised purines in the intervention group (r = 0.388, p = 0.002).

4.2.4 The impact of HbA1c on DNA damage in NIDDM subjects

NIDDM volunteers with poor glycaemic control showed significantly decreased levels (- 27%) of oxidative DNA damage after an intervention period of 4 weeks (T0: 5.41 ± 1.74, T1: 3.95 ± 0.69, T2: 4.27 ± 1.2). After further 4 weeks of intervention, levels rose a bit but were still 21.1% lower in comparison to T0. There were no significant changes in subjects with HbA1c ≤ 7%. This effect is shown in Fig. 14.

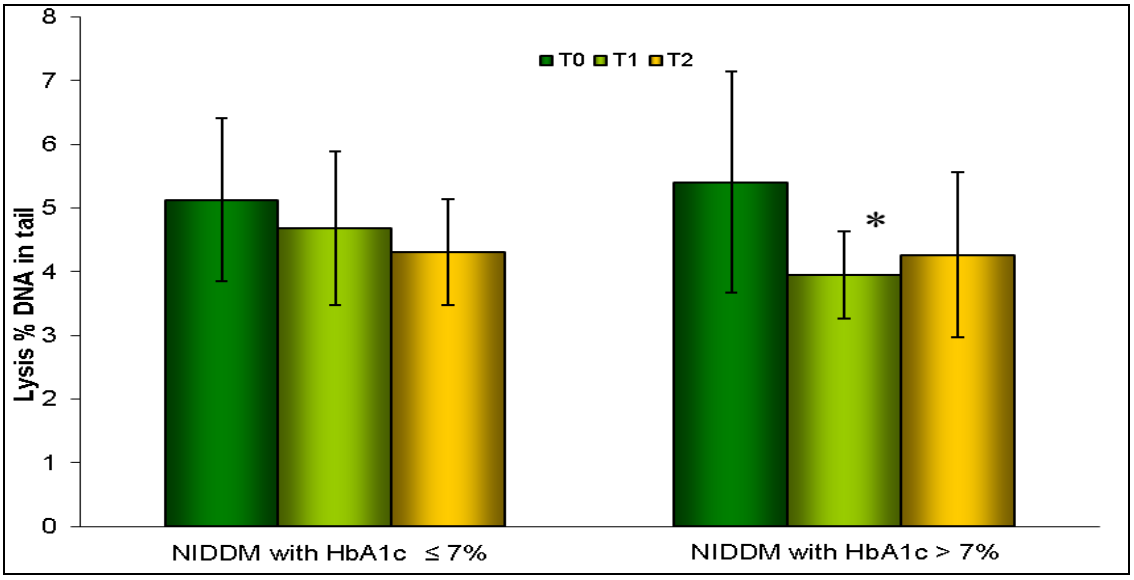


Fig. 14: Subjects with poor glycaemic control (HbA1c > 7%) showed significantly decreased levels of oxidative DNA damage after 4 weeks of intervention (p ≤ 0.05; * indicates a significant difference from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

H_2O_2 resistance of lymphocytes and levels of oxidised purines and pyrimidines showed no significant changes in NIDDM subjects during the intervention in both groups. There were no significant changes detected after return to usual diet.

4.2.5 The link between fasting plasma glucose and DNA damage

Over the time of the intervention period, subjects with lower concentrations of fasting plasma glucose (≤ 6.2 mmol/l) tended to a reduction in oxidative DNA damage after 8 weeks of intervention with - 18.18% in comparison to baseline (p ≤ 0.15; T0: 5.50 ± 1.86, T1: 4.69 ± 1.42, T2: 4.50 ± 1.52; n = 22). Participants of the intervention group with greater levels of fasting plasma glucose (> 6.2 mmol/l) showed no significantly changes in DNA damage (p ≤ 0.05; T0: 5.87 ± 2.47, T1: 4.73 ± 1.24, T2: 4.55 ± 1.12; n = 46).

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 measurement during the intervention period in subjects with lower (T3: 4.73 ±1.55) and higher (T3: 4.69 ± 1.10) fasting plasma glucose concentrations. The main reason for the insignificantly changes in oxidative DNA damage were the low number of subjects in this subgroup.

4.2.6 The link between fasting plasma glucose and DNA damage in NIDDM subjects

Levels of oxidative DNA damage changed significantly in NIDDM subjects with high levels of fasting plasma glucose (> 6.2 mmol/l). The oxidative DNA damage decreased significantly by 19.78% after 4 weeks (T1) and by 21.81% after 8 weeks (T2) of intervention when compared to baseline (T0). The levels of oxidative DNA damage retained constant in subjects with fasting plasma glucose ≤ 6.2 mmol/l. These effects are shown in Fig. 15.

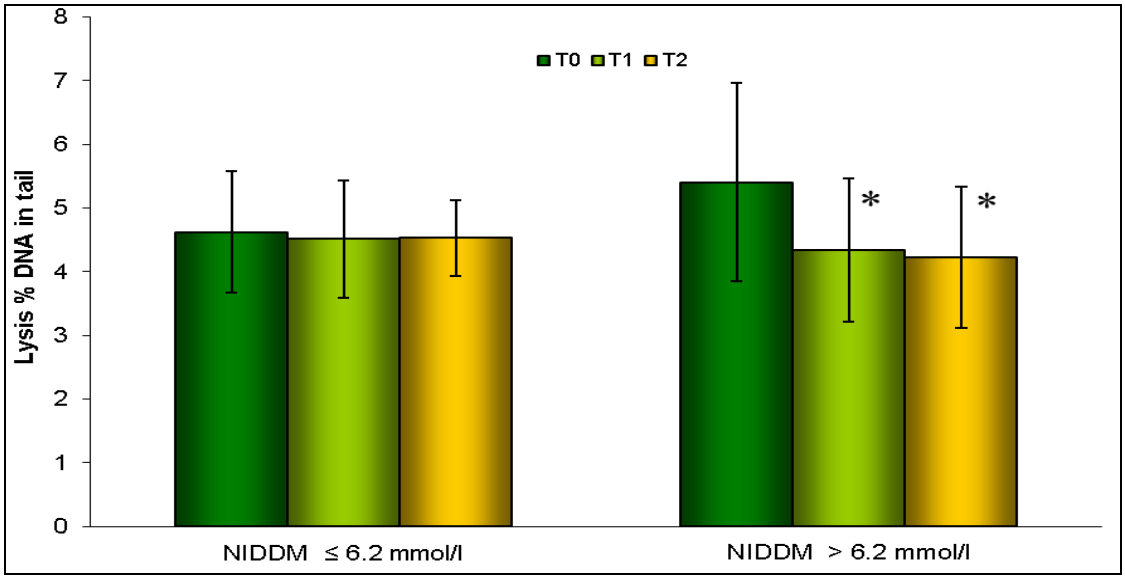


Fig. 15: NIDDM subjects with fasting plasma glucose > 6.2 mmol/l showed significantly decreased levels of oxidised DNA damage after 4 and 8 weeks of intervention ($p \leq 0.05$; * indicates a significant difference from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

Glycaemic control is assessed by HbA1c and fasting plasma glucose. Hyperglycaemia is responsible for increased oxidative stress, which is leading to higher levels of oxidative DNA damage. In summary we showed that subjects with HbA1c > 7% or fasting plasma glucose > 6.2 mmol/l have higher levels of oxidative DNA damage at baseline. Furthermore they show a much stronger response to the intervention. These

changes could be due to the change in dietary habits in combination with improved fasting plasma and HbA1c levels.

4.2.7 The association between triglycerides and DNA damage

Subjects of the intervention group with triglyceride concentrations lower or higher than 1.2 mmol/l showed a reduction (not significant) in oxidative DNA damage when compared to baseline. The group with lower amounts of triglycerides (≤ 1.2 mmol/l) 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 (> 1.2 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. These effects are shown in Fig. 16.

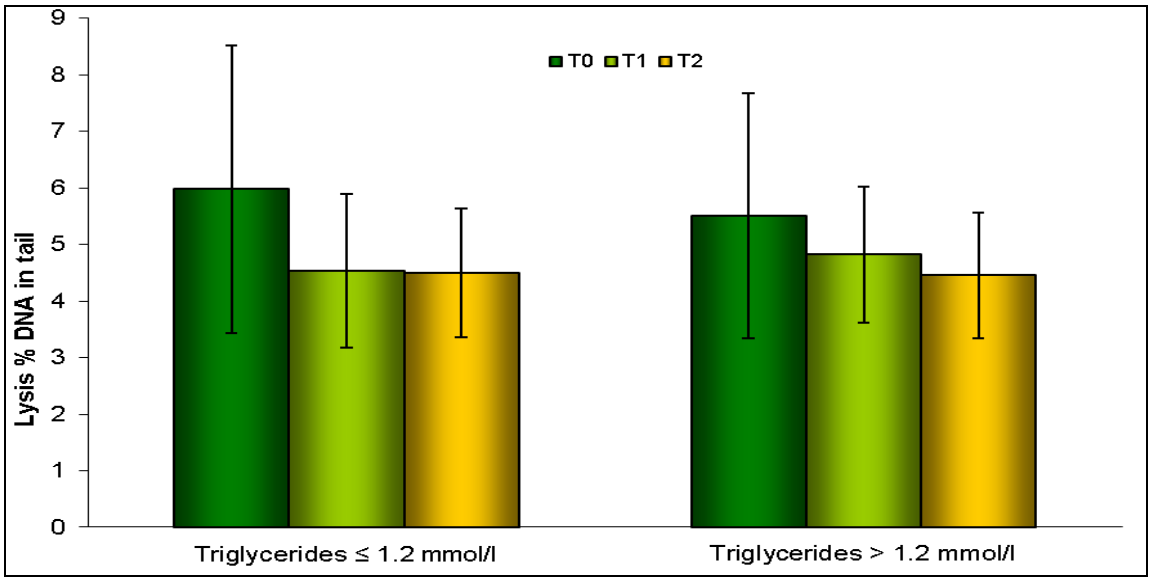


Fig. 16: Subjects with high and low levels of triglycerides showed a reduction in DNA damage (T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

The intervention showed no significant changes in 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 were observed between T3 (after return to normal diet) and T0, T1 or T2.

Our results showed high reductions in the levels of oxidative DNA damage during the intervention period when considering the triglyceride levels. Decreased levels of oxidative DNA damage were seen in both groups. However subjects with lower levels of triglycerides showed a higher reduction in DNA damage than subjects with higher

triglyceride concentrations. Although the decrease was high, the results were not significant. At baseline the SD was very high in both groups, but it 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 on NIDDM subjects in control and intervention group

Oxidative DNA damage showed a reduction with a decrease by 19.61% in NIDDM subjects with higher triglycerides (> 1.2 mmol/l) after the intervention period of 8 weeks. These results were not significant (T0: 5.15 ± 1.44, T1: 4.34 ± 1.09, T2: 4.14 ± 1.13 and T3: 4.41 ± 1.04). This is shown in Fig. 17.

NIDDM subjects with low triglycerides (≤ 1.2 mmol/l) showed no significant changes during the intervention period and after return to usual diet (T0: 4.93 ± 1.04, T1: 4.43 ± 0.94, T2: 4.93 ± 1.07 and T3: 4.71 ± 1.07).

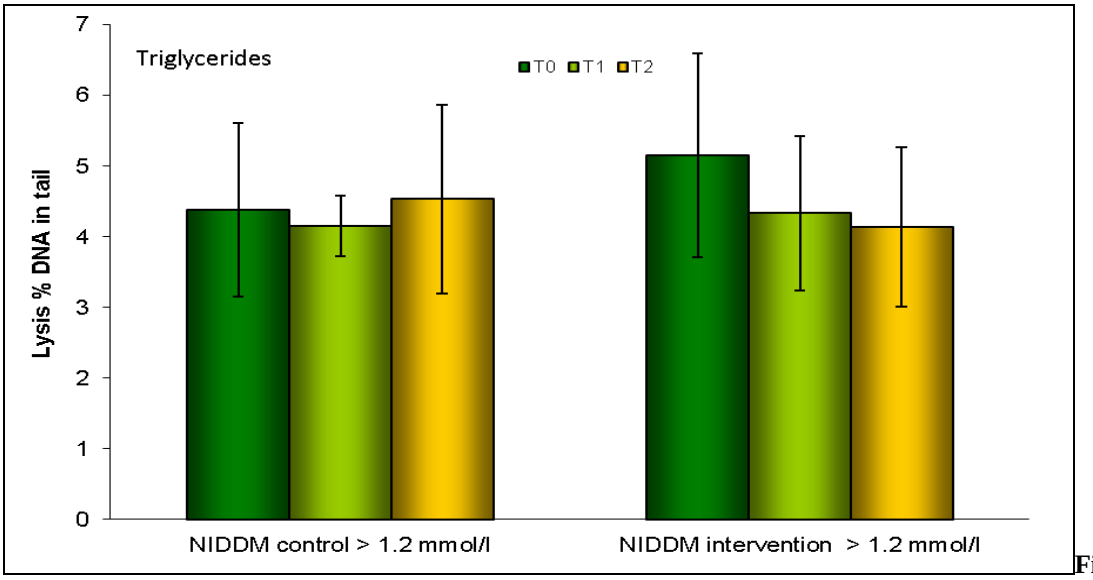


Fig. 17: NIDDM subjects with high levels of triglycerides (> 1.2 mmol/l) showed no significant effects on oxidised DNA damage in both groups (T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

4.2.9 The association of age and DNA damage

Elderly subjects (aged ≥ 60 years) of the intervention group showed significantly lower levels of oxidative DNA damage after 8 weeks (- 19.96%) when compared to baseline (T0: 5.61 ± 2.08, T1: 4.66 ± 1.34, T2: 4.49 ± 1.29 and T3: 4.71 ± 1.29).

In younger subjects (aged < 60 years) decreased levels of DNA damage were seen also after 4 weeks (- 20.12%) and after 8 weeks (- 23.67%), but these results were not significant (T0: 6.21 ± 3.09, T1: 4.96 ± 0.87, T2: 4.74 ± 0.93 and T3: 4.63 ± 0.92). The main reason for the insignificant change in this group was the high SD at baseline. This is shown in Tab. 18.

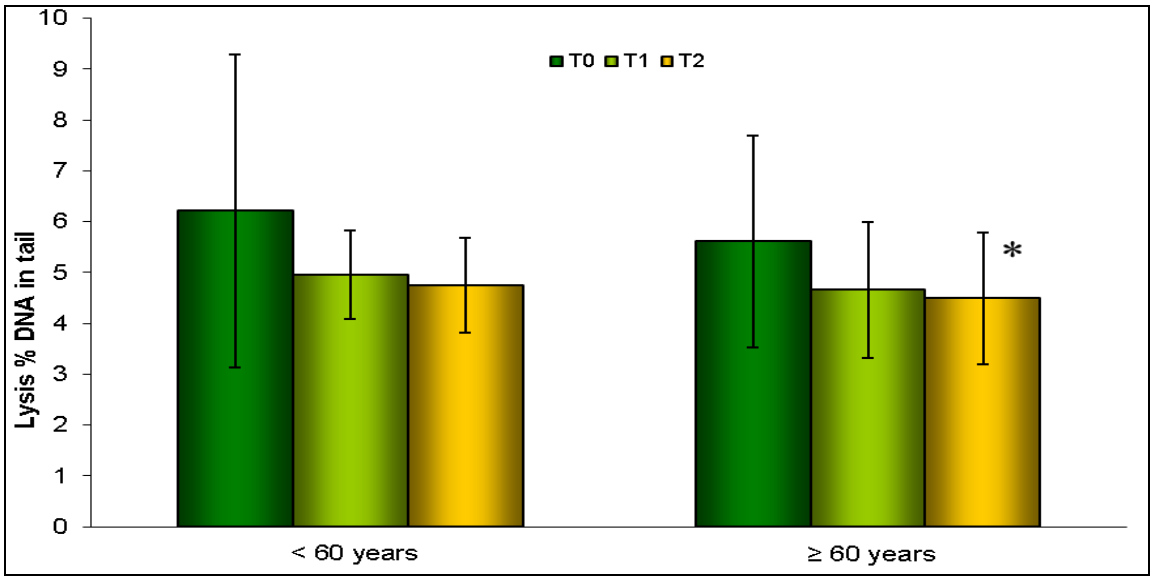


Fig. 18: Subjects aged ≥ 60 years showed significantly lower levels of DNA damage after the intervention in comparison to baseline ($p \leq 0.05$; * indicates significant differences from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

In this study, younger subjects (< 60 years) showed a significant decrease in the number of Endo III-sensitive sides 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 usual 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 effect on DNA damage.

4.2.10 **The association of age and DNA damage in NIDDM subjects**

In elderly (≥ 60 years) NIDDM subjects levels of oxidative DNA damage decreased significantly. The positive effect was detected after 4 weeks with a decrease by 18.79% and a reduction by 19.35% after 8 weeks of intervention in respect to baseline. In NIDDM subjects younger than 60 years no significant changes observed (T0: 5.27 ± 1.49, T1: 4.28 ± 1.02 and T2: 4.25 ± 1.08). This effect is shown in Fig. 19.

Fig. 20 shows the beneficial effect after 8 weeks of intervention in NIDDM subjects ≥ 60 years (control group: T0: 4.45 ± 0.66, T1: 4.48 ± 0.66 and T2: 5.22 ± 1.52; intervention group: T0: 5.27 ± 1.49, T1: 4.28 ± 1.02 and T3: 4.25 ± 1.08).

Two antioxidant supplementation studies are worth mentioning in this context. They also investigated the association between DNA damage (evaluated by the comet assay) 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].

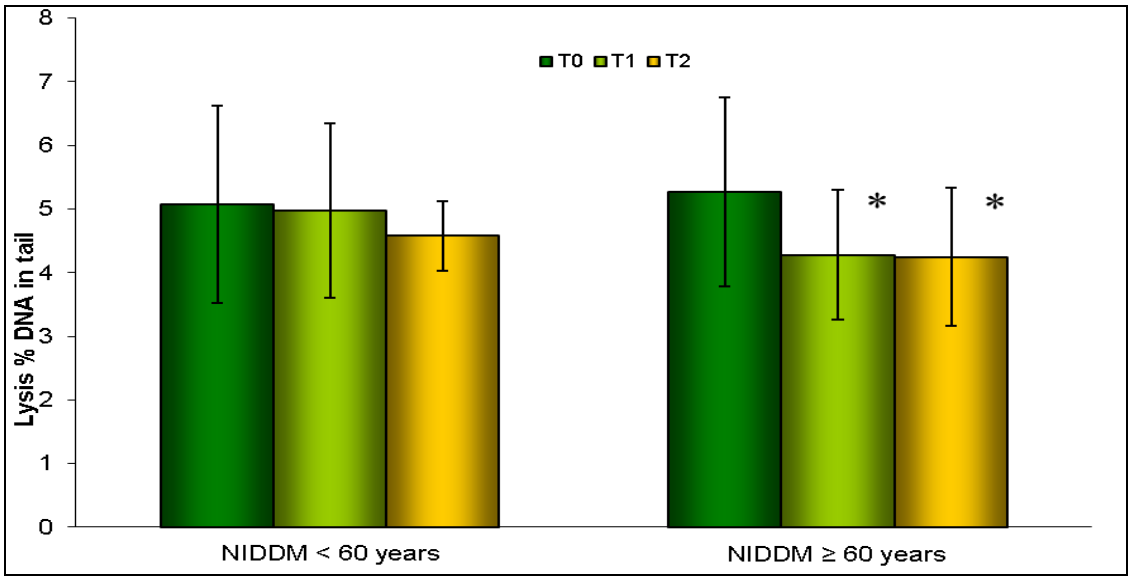


Fig. 19: NIDDM subjects aged ≥ 60 years showed significantly lower levels of DNA damage after the intervention of 4 and 8 weeks in comparison to baseline ($p \leq 0.05$; * indicates significant differences from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

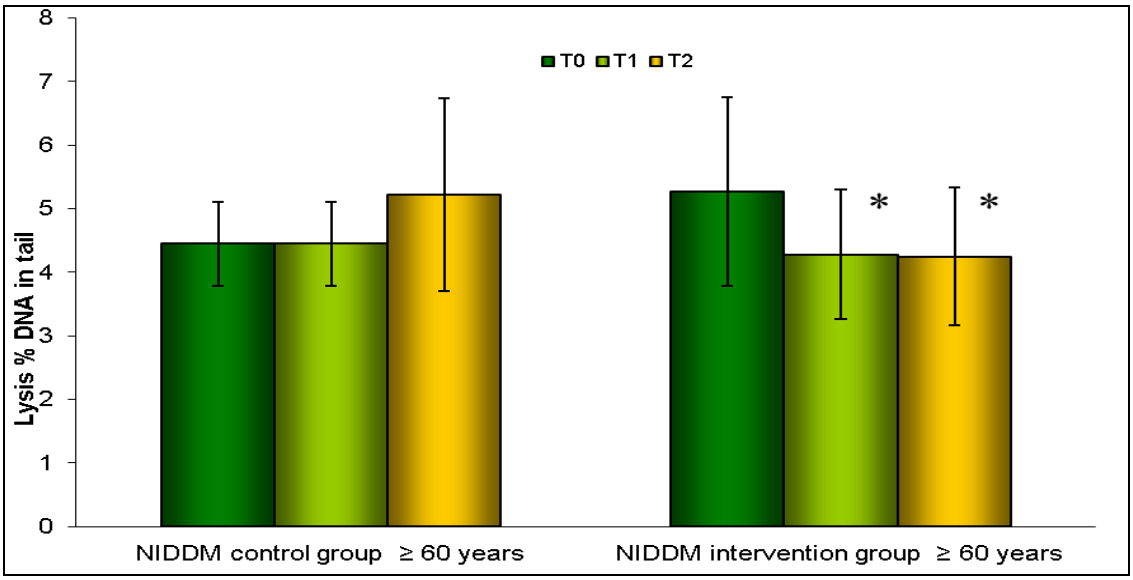


Fig. 20: NIDDM subjects aged ≥ 60 years showed significantly lower levels of DNA damage after 4 weeks and 8 weeks when compared to baseline ($p \leq 0.05$; * indicates significant differences from baseline; T0 = baseline, T1 = after 4 weeks, T2 = after 8 weeks).

4.2.11 The relation between oxidative DNA damage and vitamins

Epidemiologic studies suggest that antioxidant capacity is improved by the consumption of vegetable products, thereby decreasing the risk of developing diseases related oxidative stress [PORRINI and RISO, 2000]. The intervention with 300 g vegetables and 25 ml plant oil led to a significant increase in α-, ß-Carotene, Lutein and γ-tocopherol. In the intervention group (including IDDM-, NIDDM-, healthy- and IFG-

subjects) there were no significant correlations between changes in vitamin concentrations in plasma and changes in the levels of oxidative DNA-damage in lymphocytes.

Astley et al. investigate the effect of mixed carotene capsules, cooked minced carrots, mandarin oranges and Vitamin C tablets on DNA damage and repair. The intervention with mixed carotene capsules and minced carrots led to a significant increase in alpha and beta carotene. Intervention with carrots led to a significant improvement in repair capacity but there were no associations between increase in repair activity and changes in vitamin concentrations could be observed [ASTLEY et al., 2004].

In another study performed by Porrini et al., the effect of spinach and spinach + tomato puree on resistance to H_2O_2 induced DNA damage was investigated. The intervention led to increased resistance to H_2O_2. After intervention with spinach +tomato an increase in lutein was observed. An inverse correlation between lycopene concentration in lymphocytes and DNA damage was observed. [PORRINI et al., 2002] Riso et al. investigated the effect of tomato puree on lymphocyte resistance to oxidative stress. They concluded that tomato products can increase resistance to H_2O_2 induced DNA damage. Since the trend in relative tail moment and plasma lycopene concentrations was similar, they suggest an association between lycopene concentrations and oxidative stress [RISO et al., 1999].

In an older study by Collins et al., the carotenoids lutein, zeaxanthin, lycopene, ß-cryptoxanthin, α- and ß-carotene and vitamin E were measured in serum by HPLC. This study was looking for effects of carotenoid supplements on DNA damage compared with placebo. There were significantly increased levels in α- and ß carotene and lutein, and smaller increases in lycopene observed. When individual values of serum carotenoid concentrations were compared with the corresponding measures of lymphocytes DNA damage, negative correlations were seen. A negative correlation between endo III-sensitive sites and carotenoid concentrations was observed. Correlations were much stronger with lutein and ß-carotene than with lycopene. But there were no protective effects of carotenoid supplementation on levels of oxidative DNA damage [COLLINS et al., 1998].

Mean values of endogenous DNA damage of healthy smokers and non-smokers subjects aged 50-59 years were examined for any correlation with individual plasma antioxidant

levels by Duthie et al. A positive correlation was observed between strand breaks and α-tocopherol in non-smokers. There were weak and non-significant correlations between oxidised bases and antioxidant concentrations. These authors concluded that one has to consider other variables that affect endogenous DNA damage, including intrinsic antioxidant defences, and DNA repair [DUTHIE et al., 1996].

Porrini and Riso observed in their study an inverse correlation between plasma lycopene concentration and oxidative DNA. This indicates that when dietary antioxidant intake is consistent, plasma antioxidant concentrations reflect well the cellular antioxidant capacity even if plasma is more directly subjected to the fluctuation of dietary antioxidant intake than cells are [PORRINI and RISO, 2000].

4.2.12 **The relation between oxidative DNA damage and vitamins in NIDDM subjects**

To verify whether the level of oxidative DNA damage responded rapidly to vitamin intake, differences in DNA damage and vitamin concentrations between baseline (T0) and after 8 weeks of intervention (T2) were calculated and correlated with each other. NIDDM subjects showed a trend toward a correlation (r = -0.366, p = 0.051) between Δ Lutein and Δ Lysis. This effect is shown in Fig. 21. Another result, which is shown in Fig. 22, displays the correlation between Δ ß-carotene and Δ Lysis (r = -0.446, p = 0.015). . There were no significant results observed between any of the mentioned parameters in the control group.

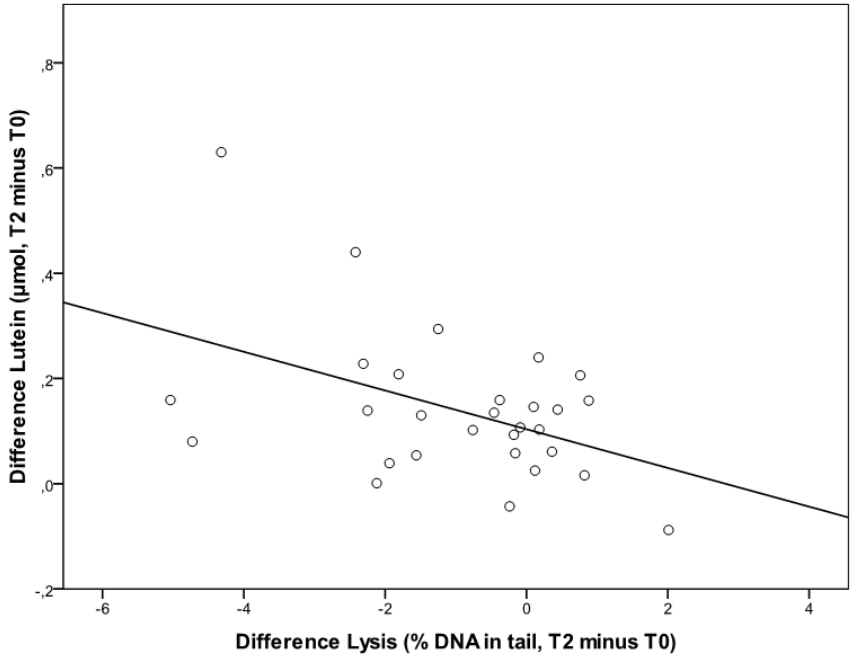


Fig. 21: Association between Δ lutein and Δ Lysis in NIDDM subjects of the intervention group (r = -0.366, p = 0.051)

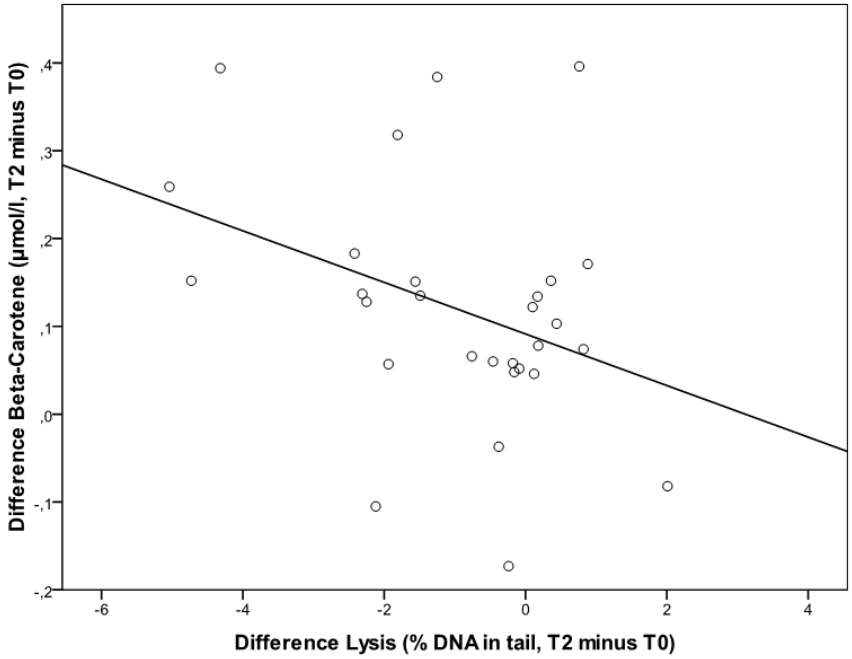


Fig. 22: Association between Δ ß-carotene and Δ Lysis in NIDDM subjects of the intervention group (r = -0.446, p = 0.015).

Sampson et al. investigated the effect of 400 I.U. α-Tocopherol on DNA and LDL oxidative susceptibility in subjects with T2DM. They found a direct relationship between DNA oxidative susceptibility and plasma α-tocopherol levels at baseline, but the supplementation had no impact on DNA and LDL oxidative susceptibility [SAMPSPON et al., 2001].

5 Conclusion

The consumption of 300 g mixed vegetables and 25 ml plant oil per day led to significant changes in the amount of oxidative DNA damage in the whole study group. The protective effect of the intervention was seen in participants with diabetes type 2, especially in NIDDM subjects.

In association to their stage of health, the study groups displayed no differences in the levels of DNA damage at baseline. Before the intervention started, recruited volunteers with high fasting glucose (> 6.2 mmol/l) showed significantly higher levels of oxidised purines when compared to participants with normal fasting glucose (≤ 6.2 mmol/l). They also displayed a significant correlation between C-peptide and resistance to H_2O_2-induced DNA damage at baseline.

After the intervention period the total study group showed significantly lower levels of DNA damage when compared to baseline. In subjects with HbA1c > 7% levels of DNA damage were significantly decreased after 4 and 8 weeks of intervention and subjects with ≤ 7 % displayed significantly lower levels of DNA damage after 8 weeks of intervention. H_2O_2-induced oxidative DNA damage was significantly reduced in subjects with HbA1c > 7% after 8 weeks of intervention.

Subjects with NIDDM displayed significantly reduced DNA damage after 4 and 8 weeks in comparison to baseline. NIDDM subjects with levels of HbA1c > 7% and fasting plasma glucose > 6.2 mmol/l showed significant changes of oxidative DNA damage after 8 weeks of intervention. Significant correlations between Δ HbA1c and DNA damage indicate that better glycaemic control leads to a decreased amount of oxidative DNA damage in diabetics. The DNA damage in NIDDM subjects with triglyceride concentrations > 1.2 mmol/l was insignificantly decreased with a reduction of 19.61%. Levels of oxidative DNA damage were significant reduced in elderly subjects with NIDDM after 4 and 8 weeks of intervention. The intervention with mixed vegetables and plant oil rich in AOs led to a significant higher concentrations in α-, ß-carotene, lutein and γ-tocopherol in the intervention group. In participants with NIDDM a correlation between change in DNA strand breaks (Lysis) and change in concentration of ß-carotene ($p < 0.05$) and lutein ($p = 0.051$) were observed.

These present results are underlining the beneficial effects of a diet rich in vegetables and plant oil rich in PUFAs especially in people suffering from T2DM.

6 Abstract

At the Department of Nutritional Sciences in Vienna, a randomized controlled intervention study was performed to measure the impact of a nutritional intervention on the levels of oxidative damage in T2DM. For the DIAPLANT study three physicians working at Vienna Health Centre South recruited 76 T2DM subjects (41 non-insulin-dependent (NIDDM) subjects and 35 insulin dependent T2DM subjects (IDDM-T2)), 12 subjects with impaired fasting plasma glucose and 11 healthy subjects. The participants were randomly assigned to the control or intervention group. The control group received detailed nutritional information about the protective effects of antioxidants and the benefits of polyunsaturated fatty acids. The intervention group received 300 g mixed vegetables and 25 ml plant oil per day. The subjects were requested not to make any additional changes in dietary habits and lifestyle during the study period. Blood samples were taken to measure the amount of the DNA damage at baseline, after 4 weeks, after 8 weeks and after 16 weeks (after returning to normal eating habits). Oxidative DNA damage was measured in lymphocytes by using comet assay. By using this method, not only single and double strand breaks, but also oxidised bases (purine and pyrimidine) and resistance to H_2O_2-induced DNA damage was measured. In order to display a more accurate view, possible influencing parameters such as fasting blood glucose, triglycerides, age, ß-carotene and lutein which have increased influence on the level of DNA damage, were included in the statistical evaluation of results.

The results show that the daily consumption of vegetables and high-quality plant oil rich in vitamins and antioxidants leads to decreased levels of oxidative DNA damage compared to the measurements before the start of the trial in T2DM subjects. The intervention showed significantly decreased levels of DNA damage in the total study group after 8 weeks and in NIDDM subjects also after 4 weeks in comparison to baseline. A slight but not significant increase in the resistance to H2O2-induced DNA damage was also found in the total study group. Levels of oxidised bases did not change significantly during the intervention. There were no significant changes in any marker of oxidative DNA damage in the control group. Furthermore we could show that HbA1c has a significant impact on DNA damage. Poor controlled volunteers with HbA1c > 7% had significantly lower levels of DNA damage after 4 and 8 weeks of intervention in

comparison to baseline. Better controlled subjects with HbA1c < 7% displayed significant reductions after 8 weeks of intervention. Significant correlations between better glucose control and oxidative DNA damage underscore the association between these two parameters. We also measured significant decreased levels of oxidative DNA damage in NIDDM subjects with fasting plasma glucose > 6.2 mmol/l after 4 weeks and 8 weeks of intervention. DNA damage tended to decrease in NIDDM subjects with triglyceride concentrations > 1.2 mmol/l. In elderly subjects ≥ 60 years significant lower levels of oxidative DNA damage were measured after 4 weeks and they decreased even more after 8 weeks of consumption when compared to baseline. Participants with NIDDM < 60 years showed no significant changes during the intervention. The intervention led to significant increased concentrations in α-, ß-carotene, lutein and γ-tocopherol in plasma. The results showed no significant associations between changes in vitamin concentrations and changes in the levels of DNA damage in the intervention group. But NIDDM subjects displayed a significant association between Δ lutein and Δ oxidative DNA damage after the intervention period.

In conclusion, the results of our study showed that a diet rich in antioxidants and polyunsaturated fatty acids from natural source has positive impact on levels of oxidative DNA damage in subjects with T2DM.

7 Zusammenfassung

Am Institut für Ernährungswissenschaften in Wien wurde eine randomisierte Interventionsstudie durchgeführt, um den Einfluss einer Ernährungsintervention auf das Ausmaß an oxidativen Schäden bei T2DM zu messen.

Für die Studie Diaplant wurden 41 nicht-insulinpflichtige und 35 Insulinpflichtige T2DM Probanden (gesamt 76 T2DM), 12 Teilnehmer mit erhöhter Nüchternblutglukose sowie 11 Gesunde rekrutiert und zufällig zwei Gruppen zugewiesen. Die Kontrollgruppe erhielt Information über die positiven Eigenschaften von Antioxidantien und mehrfach ungesättigten Fettsäuren. Die Interventionsgruppe konsumierte täglich 300 g gemischtes Gemüse und 25 ml Pflanzenöl und wurde angewiesen das Ernährungsverhalten oder den Lebensstil während der gesamten Studie nicht zusätzlich zu ändern. Um das Ausmaß an DNA–Schäden zu messen, wurde zu Beginn der Studie, nach 4 Wochen, nach 8 Wochen sowie nach insgesamt 16 Wochen (nach Rückkehr zur normalen Essensgewohnheit) Blut abgenommen. Aus dem Frischblut wurden die Lymphozyten isoliert, und für die Detektion von DNA Strangbrüchen mittels Comet Assay herangezogen. Neben dem Gehalt an Einzel und Doppelstrangbrüchen wurden auch oxidierter Basen (Purin und Pyrimidin) und die Widerstandsfähigkeit der Lymphozyten gegenüber Wasserstoffperoxid gemessen.

Um einen genaueren Einblick darstellen zu können, wurden mögliche Einflussparameter wie Nüchternblutglukose, Triglyzeride, Alter, ß-Carotinoide und Lutein welche vermehrten Einfluss auf den Gehalt von DNA-Schäden haben können, in die statistische Auswertung mit einbezogen. Wir konnten zeigen, dass der tägliche Konsum von hochwertigem Pflanzenöl und Gemüse, reich an Vitaminen und Antioxidantien bei T2DM zu einer Reduktion des Ausmaßes an oxidativen DNA-Schäden führt. Die Intervention zeigte eine signifikante Reduktion an DNA-Schäden im Gesamtkollektiv nach 8 Wochen und in der Gruppe der NIDDM Probanden bereits nach 4 Wochen im Vergleich zum Ausgangswert vor Beginn der Intervention. Eine leichte, aber nicht signifikante Steigerung der Resistenz gegenüber Wasserstoffperoxid konnte im Gesamtkollektiv gemessen werden. Der Gehalt an oxidierten Basen änderte sich während der Intervention nicht. Es wurden keine signifikanten Änderungen bezüglich der untersuchten Parameter und Veränderungen der DNA-Schäden in der Kontrollgruppe festgestellt. Bei schlecht eingestellten Probanden mit einem HbA1c von

> 7% wurde bereits nach kurzer Interventionsperiode eine signifikante Abnahme der DNA-Schäden erzielt im Vergleich zu Beginn der Studie. Gut eingestellte T2DM Probanden mit einem HbA1c von ≤ 7% zeigten erst nach längerer Interventionszeit von 8 Wochen eine signifikante Reduktion. Signifikante Korrelationen zwischen Δ HbA1c und Δ DNA Schaden zeigten, dass eine Verbesserung der Blutzuckerwerte zu einer Reduktion der DNA-Schäden führen kann. Eine signifikante Abnahme von DNA-Schäden konnte auch bei NIDDM Probanden mit erhöhter Nüchtern-Blutglukose-Konzentration > 6.2 mmol/l bereits nach 4 Wochen und fortwehrend nach 8 Wochen gemessen werden. Eine leichte, aber nicht signifikante Reduktion wurde bei NIDDM Probanden mit erhöhter Konzentration an Triglyzeriden > 1.2 mmol/ festgestellt. Auch bei älteren Studienteilnehmern ≥ 60 Jahre mit NIDDM wurde eine Reduktion an oxidativen DNA-Schäden bereits nach 4 Wochen und noch weiter nach 8 Wochen im Vergleich zum Ausgangswert vor Beginn der Intervention erzielt. Studienteilnehmer < 60 Jahren zeigten keine signifikanten Entwicklungen während der Ernährungsintervention. Die Intervention führte im Plasma zu einem erhöhten Anstieg von α-, ß-Carotinoide, Lutein und γ- Tocopherol. Während die Interventionsgruppe im gesamten keine signifikanten Korrelationen zwischen dem Anstieg der Vitaminkonzentration im Plasma und der Reduktion von DNA Schäden zeigte, wurde bei Probanden mit NIDDM eine signifikante Korrelation zwischen Δ Lutein und Δ Lyse gemessen. Zwischen Δ ß-Carotin und oxidativen DNA-Schaden konnte ein Trend eines Zusammenhangs festgestellt werden (P <0,06).

Zusammenfassend, konnte im Rahmen dieser Studie gezeigt werden, dass eine Ernährungsintervention mit Gemüse und hochwertigem Pflanzenöl positiven Einfluss auf oxidative DNA Schäden bei T2DM hat.

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Erklärung zur gemeinsamen Bearbeitung eines Themas

Gemäß dem Universitätsgesetz §81(Abs. 3 UG) wurden die Diplomarbeiten mit dem Titel

„Influence of a Dietary Intervention on oxidative DNA damage in Non-insulin-dependent Diabetes Mellitus Type 2"

und dem zweiten Titel

"Influence of a Dietary Intervention on oxidative DNA damage in Insulin-dependent Diabetes Mellitus Type 2"

von Christiane Schiermayr und Simone Pleifer im Rahmen einer Ernährungsinterventionsstudie am Institut für Ernährungswissenschaften unter der Betreuung von Herrn Ao. Univ.-Prof. Dr. Karl-Heinz Wagner und Frau Mag. Elisabeth Müllner an der Universität Wien gemeinsam verfasst.

Im Inhaltsverzeichnis dieser Arbeit sind die Teile der jeweiligen Verfasserin genau zugeordnet.

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