universität wien

DISSERTATION

Titel der Dissertation

DNA damage and genomic stability in diabetic and non-diabetic individuals and the impact of a dietary intervention

Verfasserin

Mag. Elisabeth Müllner

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr.rer.nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 091 474
Dissertationsgebiet lt. Studienblatt:	Ernährungswissenschaften
Betreuerin / Betreuer:	Univ.-Prof. Dr. Karl-Heinz Wagner

I Abstract

The number of people suffering from diabetes has reached an alarming level and is predicted to increase even further. This trend is due to population growth, ageing, unhealthy diet and lack of physical activity. Type 2 diabetes (T2DM) is a multifactorial disease characterized by high blood glucose levels resulting from insulin resistance and impaired insulin secretion. It is well recognized that T2DM is associated with increased risk for micro- and macrovascular complications. Furthermore, recent epidemiological studies suggest increased cancer risk in patients with T2DM compared with healthy individuals.

The present study aimed to assess whether T2DM is associated with increased oxidative damage to DNA and genomic instability and whether a healthy diet, including 300 g of vegetables and 25 ml polyunsaturated fatty acid (PUFA)-rich walnut oil as replacement for saturated fatty acids (SFA), can improve oxidative damage to DNA and genome damage rate in diabetic and non-diabetic individuals.

Seventy-six diabetic and 21 non-diabetic individuals participated in this study and were randomly assigned to either an “intervention” or an “information only” group. All participants received information about the benefits of a healthy diet, while subjects of the intervention group additionally received 300 g of vegetables and 25 ml PUFA-rich plant oil per day for 8 weeks. Blood samples were taken at baseline, as well as after 4, 8 (end of intervention), and 16 weeks.

To assess dietary compliance plasma or red cell concentrations of vitamins, antioxidants (vitamins A, E, K, B12, folic acid and carotenoids) and fatty acids were measured. Oxidative damage to DNA and oxidised purines (sites sensitive to formamidopyrimidine DNA glycosylase (FPG)) in mononuclear cells were measured by comet assay, chromosomal damage in lymphocytes and buccal cells by micronucleus (MN) cytome assay. Urinary excretion of 8-oxo-7,8-dihydro-2’-deoxy-guanosine (8-oxodG, reflects global DNA oxidation) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo, reflects global RNA oxidation) were measured by ultraperformance liquid chromatography and tandem mass spectrometry. Furthermore, levels of fasting plasma glucose and glycosylated haemoglobin (HbA1c) were assessed.

The comparison of chromosomal damage in buccal cells between diabetic and non-diabetic individuals at baseline revealed significantly higher MN levels in diabetic individuals. Subjects in the highest tertile of waist circumference, fasting plasma glucose, HbA1c and cardiovascular disease (CVD) risk (assessed by the Framingham general cardiovascular risk score) had

significantly higher buccal cell MN frequency compared to participants in the lowest tertile. Furthermore, levels of FPG-sensitive sites and 8-oxoGuo in urine were significantly higher in diabetic individuals compared to non-diabetic participants. In contrast levels or urinary 8-oxodG, oxidative DNA strand breaks and chromosomal damage in lymphocytes did not differ between diabetic and non-diabetic individuals. However, diabetic individuals with lymphocyte MN frequencies above the 50th percentile had significantly higher levels of fasting plasma glucose, HbA1c and were at higher risk for CVD compared with diabetic individuals in the low 50th percentile. Non-diabetic individuals with MN frequencies above the 50th percentile had significantly lower vitamin B12 levels compared to their healthy counterparts.

The intervention with vegetables and PUFA-rich plant oil led to a significant increase in plasma antioxidant and vitamin concentrations (vitamins E, K, folic acid and carotenoids) and essential fatty acids (linoleic- and linolenic acid) that are predominant in the intervention oil. Diabetic individuals of the intervention group showed a significant reduction in HbA1c and DNA strand breaks. Levels of HbA1c were also improved in diabetic patients of the information group, but oxidative damage to DNA was not altered. Levels of chromosomal damage in lymphocytes were not changed, only apoptosis was slightly increased. In buccal cells, the number of binucleated cells, karyorrhexis and condensed chromatin was significantly reduced, in both, the intervention and information group. The number of pycnotic cells was only reduced in participants of the intervention group, and therefore changes were significantly different between the two treatment groups. Urinary 8-oxodG and 8-oxoGuo excretion remained unchanged in both groups.

This study provides evidence that a healthy diet reduces levels of DNA strand breaks in diabetic individuals while MN frequency, as a marker of chromosomal damage, is not altered. Interactions between glycemic control, CVD-risk and genomic damage can be suggested.

II Zusammenfassung

Die Zahl der an Diabetes erkrankten Menschen hat ein alarmierendes Ausmaß erreicht und ist weiter im Steigen begriffen. Faktoren wie Wachstum und Alterung der Bevölkerung aber auch ungesunde Ernährung und mangelnde körperliche Aktivität werden für diese Entwicklung verantwortlich gemacht. Typ 2 Diabetes (T2DM) ist eine multifaktorielle Erkrankung, charakterisiert durch erhöhte Blutglukose-Konzentrationen, welche durch Insulinresistenz und Insulinmangel bedingt sind. Diabetes geht mit einem erhöhten Risiko für mikro- und makrovaskuläre Komplikationen einher. Im Rahmen epidemiologischer Studien wird auch ein erhöhtes Krebsrisiko bei T2DM Patienten im Vergleich zu Gesunden diskutiert.

Das Ziel der vorliegenden Arbeit war es zu untersuchen, ob Diabetes mit einem erhöhtem Ausmaß an oxidativen DNA Schäden bzw. chromosomalen Schäden assoziiert ist, und ob eine Ernährungsintervention, reich an Gemüse und mehrfach ungesättigten Fettsäuren (PUFA) als Ersatz für gesättigte Fettsäuren (SFA), diese Parameter verbessern kann.

Sechsundsiebzig Diabetiker sowie 21 Nicht-Diabetiker nahmen an der Studie teil und wurden randomisiert der „Interventions-“ oder „Informationsgruppe“ zugeteilt. Allen Probanden wurde die Bedeutung und der gesundheitliche Nutzen einer gesunden Ernährung näher gebracht, wobei Probanden, welche der Interventionsgruppe zugeteilt worden waren, zusätzlich 300 g Gemüse und 25 ml Walnussöl täglich für 8 Wochen erhielten. Blutabnahmen fanden zu Beginn der Studie, sowie nach 4, 8 (Ende der Intervention) und 16 Wochen statt.

Zur Compliance-Überprüfung wurden Vitamine und Antioxidantien (Vitamine A, E, K, B12, Folsäure und Carotinoide) im Plasma oder in Erythrozyten sowie das Fettsäuremuster gemessen. Oxidative DNA Schäden und oxidierte Purine (FPG (formamidopyrimidine DNA glycosylase) sensitive sites) in Einkernzellen wurde mittels Comet Assay, chromosomale Schäden in Lymphozyten und Mundschleimhautzellen mittels Micronucleus Cytome Assay erfasst. 8-oxo-7,8-dihydro-2’-deoxyguanosine (8-oxodG, widerspiegelt DNA Oxidation) und 8-oxo-7,8-dihydroguanosine (8-oxoGuo, widerspiegelt RNA Oxidation) wurden im Urin mittels UPLC-MS/MS gemessen. Weiters wurde der nüchtern Blutzuckerspiegel und glycosyliertes Hämoglobin (HbA1c) gemessen.

Diabetiker wiesen ein signifikant höheres Ausmaß an chromosomalen Schäden in Mundschleimhautzellen im Vergleich zu Nicht-Diabetikern auf. Zudem hatten Probanden im höchsten Tertil von Bauchumfang, Blutzucker, HbA1c, des Risikos für kardiovaskuläre

Erkrankungen (berechnet mittels Framingham general cardiovasuclar risk score) eine signifikant höhere Anzahl an Mikrokernen in Mundschleimhautzellen im Vergleich zu Probanden im niedrigsten Tertil. Die Konzentration an FPG-sensitive sites und 8-oxoGuo im Harn war signifikant höher bei Diabetikern im Vergleich zu Nicht-Diabetikern.

Die beiden Gruppen wiesen ein ähnliches Ausmaß an oxidativen DNA Schäden im Harn, oxidativen DNA Strangbrüchen und chromosomalen Schäden in Lymphozyten auf. Allerdings hatten Diabetiker mit einem hohen Anteil an Mikrokernen in Lymphozyten (über der 50. Perzentile) signifikant höhere nüchtern Blutzucker-, HbA1c-Konzentrationen und ein höheres Risiko für kardiovaskuläre Erkrankungen im Vergleich zu Diabetikern mit einer Anzahl an Mikrokernen unter der 50. Perzentile. Nicht-Diabetiker mit einer Lymphozyten-Mikrokern-Rate über der 50. Perzentile hatten signifikant niedrigere Vitamin B12-Spiegel als Nicht-Diabetiker unter der 50. Perzentile.

Die Intervention mit Gemüse und PUFA-reichem Pflanzenöl führte zu einem signifikanten Anstieg an Antioxidantien, Vitaminen (Vitamine E, K, Folsäure, Carotinoide) und essentiellen Fettsäuren, die im Pflanzenöl vorherrschend waren (Linol- und Linolensäure). Lediglich Vitamin B12 sank signifikant nach 8-wöchiger Intervention. Die Intervention führte bei Typ 2 Diabetikern zu einer signifikanten Reduktion von HbA1c und der Zahl an DNA-Strangbrüchen. Auch in der Informationsgruppe sank der HbA1c-Wert signifikant, das Ausmaß an DNA-Strangbrüchen änderte sich allerdings nicht. Die Intervention zeigte keinen Einfluss auf chromosomale Schäden in Lymphozyten, lediglich die Apoptoserate stieg leicht, aber signifikant an. In den Mundschleimhautzellen sank die Zahl an Zweikernzellen, sowie das Auftreten von Karryorrhexis und kondensiertem Chromatin in der Informations- als auch in der Interventionsgruppe. Die Zahl an pyknotischen Zellen sank nur in der Interventionsgruppe, weshalb die Veränderung binnen 8 Wochen signifikant unterschiedlich zwischen den beiden Gruppen war. Levels an 8-oxodG und 8-oxoGuo im Harn blieben unverändert während der Studie.

Im Rahmen dieser Studie zeigte sich, dass eine 8-wöchige Ernährungsintervention das Ausmaß an oxidativen DNA Strangbrüchen bei Diabetikern reduziert, während chromosomale Schäden unbeeinflusst bleiben. Interaktionen zwischen glykämischer Kontrolle, CVD-Risiko und chromosomalen Schäden wurden beobachtet.

III Contents

IV List of Publications

The present thesis is based on the following articles:

Paper I MÜLLNER, E., BRATH, H., PLEIFER, S., SCHIERMAYR, C., BAIERL, A., WALLNER, M., FASTIAN, T., MILLNER, Y., PALLER, K., HENRIKSEN, T., POULSEN, H. E., FORSTER, E. & WAGNER, K.-H. 2012. Vegetables and PUFA-rich plant oil reduce DNA strand breaks in individuals with type 2 diabetes. *Molecular Nutrition & Food Research;* 57, 328-338

Paper II MÜLLNER, E., BRATH, H., TOFERE, D., ADRIGAN, S., BULLA, M.-T., STIEGLMAYER, R., WALLNER, M., MAREK, R., WAGNER, K.-H. 2013. Genome damage in peripheral blood lymphocytes of diabetic and non-diabetic individuals after intervention with vegetables and plant oil. *Mutagenesis*; 28, 205-211

Paper III MÜLLNER, E., BRATH, H., NERSESYAN, A., NITZ, M., PETSCHNIG, A., WALLNER, M., KNASMÜLLER, S., WAGNER, K.-H. 2012. Nuclear anomalies in exfoliated buccal cells in healthy and diabetic individuals and the impact of a dietary intervention. *Manuscript, submitted*

V Abbreviations

ADA	American Diabetes Association
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
BE	Broken eggs
BMCyt assay	Buccal micronucleus cytome assay
BMI	Body mass index
BN	Binucleated cells
CBMN assay	Cytokinesis-block micronucleus assay
CC	Condensed chromatin
CVD	Cardiovascular diseases
DAG	Diacylglycerol
DCCT	Diabetes Control and Complication Trial
DPP-IV inhibitor	Dipeptidyl peptidase IV
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
$FADH_2$	Flavin adenine dinucleotide
FFA	Free fatty acid
Fruc-6-P	Fructose-6-phosphate
FFQ	Food frequency questionnaire
FPG	Formamidopyrimidine DNA glycosylase
GFAT	Glutamine: fructose-6-phosphate amidotransferase
GH	Growth hormone
GHR	Growth hormone receptor
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide-1
GSH	Reduced glutathione
HbA1c	Glycated haemoglobin
HDL	High density lipoprotein
HIFIα	Hypoxia-inducible factor 1α
HOMA-IR	Homeostasis model assessment of insulin resistance

HPLC	High-performance liquid chromatography
IFG	Impaired fasting glucose
IGF	Insulin growth factor
IGF-1R	Insulin growth factor 1 receptor
IGRBP	Insulin growth factor binding protein
IGT	Impaired glucose tolerance
IL-6	Interleukin 6
IR	Insulin receptor
ITDM2	Insulin-treated type 2 diabetes mellitus
KL	Kariolysis
KR	Karryorrhexis
LDL	Low density lipoprotein
MCP-1	Monocyte chemoattractant protein-1
MN	Micronucleus
MODY	Maturity onset diabetes of the young
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBuds	Nuclear buds
NDI	Nuclear division index
NEFAs	Non-esterified fatty acids
NFκB	Nuclear factor kappa B
NGSP	National Glycohaemoglobin Standardization Program
NIDDM	Non-insulin dependent type 2 diabetes mellitus
NO	Nitric oxide
NPBs	Nucleoplasmic bridges
OGGT	Oral glucose tolerance test
OGT	*O*-GlcNAc transferase
P	Pycnotic cells
PAI-1	Plasminogen activator inhibitor-1
PAR	Polymers of ADP-ribose
PARP	Poly(ADP-ribosyl) polymerase
PHA	Phytohemagglutinin
PKC	Protein kinase C

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PPARγ	Peroxisome proliferator-activated receptor γ
PUFA	Polyunsaturated fatty acid
QLQ	Quality of life questionnaire
RAGE	AGE receptor
ROS	Reactive oxygen species
SCGE assay	Single cell gel electrophoresis assay
SFA	Saturated fatty acid
SHG	Sex hormone binding globulin
Sp1	Specific Protein 1
Stat3	Signal transducer and activator of transcription 3
TC	Total cholesterol
TG	Triglycerides
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
T2DM	Type 2 diabetes
TZDs	Thizolidinediones
UDP-GlcNAc	Uridine diphosphate-N-acetyl glucosamine
8-oxodG	8-oxo-7,8 –dihydro-2’-deoxyguanosine
8-oxoGuo	8-oxo-7,8-dihydroguanosine

VI Tables

VII Figures

1 Introduction

1.1 The global burden of diabetes – present and future

Diabetes is a growing health care problem worldwide. In 2011 the estimated number of diabetes cases among adults was 366 million and is expected to rise to 552 million by 2030. Most of diabetic patients live in low- and middle-income countries and the increase in prevalence is predicted to be greatest in these countries. For Africa, an increase by 90.5% until 2030 is expected. The Western Pacific region will continue to have the highest number of diabetes cases, due to the high prevalence in China (2011: 90 million, 2030: 129.7 million) and India (2011: 61.3 million, 2030: 101.2 million) (Whiting et al., 2011). The number of people with diabetes is suggested to increase as a consequence of population growth, ageing, increasing urbanization and lifestyle changes associated with risk factors such as obesity (Danaei et al., 2011, Whiting et al., 2011). In Europe diabetes prevalence is estimated at 8.4%, whereas it accounts for 9.97% in Austria (comparative prevalence - age-standardized to the world population) (IDF, 2012).

Ninety percent of diabetic patients have type 2 diabetes (T2DM) (Massó González et al., 2009). Type 2 diabetes used to be considered as an adult-onset disease. However, the rising prevalence of childhood obesity and overweight (4.2% in 1990, 6.7% in 2010 and 9.1% in 2020 (de Onis et al., 2010)) is followed by an increase in T2DM prevalence among children and adolescents (Hu, 2011, Molnár, 2004). Currently the greatest number of people with diabetes is between 40 to 59 years of age, although there are considerable variations according to the countries' income status. In countries classified as high-income countries, most people with diabetes are over 60 years of age, while in low- and middle-income countries most people with diabetes are between 40 and 60 years (Whiting et al., 2011).

Diabetes is also an increasing economic burden. Globally, 12% of the world's total health expenditures were attributed to diabetes in 2010, which is equal to USD 376 billion. Expenditures are expected to rise to USD 490 billion in 2030. Health expenditures will not be evenly distributed among different age groups and countries. Ninety-one percent of expenditures will be spent in developed countries, while only 9% are going to be available in developing countries (Zhang et al., 2010).

4.8 million of deaths were attributable to diabetes in 2012 (IDF, 2012). Cardiovascular diseases (CVD) are the leading cause of death (60%) (Van Dieren et al., 2010), 40% of deaths are suggested to be attributable to nonvascular conditions, including 10% attributable to deaths from cancer

(Seshasai et al., 2011). Furthermore, diabetes is associated with premature mortality, suggesting that a 50-year old with diabetes dies, on average, 6 years earlier compared to a person free of diabetes (Seshasai et al., 2011).

1.2 Classification

Diabetes is a complex metabolic disorder characterized by hyperglycaemia caused by defective insulin action, insulin secretion, or both. Based on the American Diabetes Association (ADA) diabetes can be classified into 4 subgroups (ADA, 2012):

1. **Type 1 diabetes**

This form accounts for 5-10% of diabetes cases, results from an autoimmune-mediated destruction of the pancreatic β-cells and leads to absolute insulin deficiency. It commonly occurs in childhood and adolescence, although an onset in later stage of life is possible as well. Autoimmune destruction of β-cells is related to both environmental and genetic factors, which are still poorly defined (ADA, 2012).

A minority of patients with type 1, most commonly seen in African American individuals, suffers from a form of type 1 diabetes ("idiopathic" diabetes) with no evidence of autoimmunity. However, aetiologies are not known (Piñero-Piloña and Raskin, 2001).

2. **Type 2 diabetes**

Ninety to ninety-five per cent of diabetic individuals have T2DM. This form is characterized by insulin resistance combined with relative insulin deficiency. Most patients suffering from T2DM are obese or have increased abdominal fat distribution. Type 2 diabetes is associated with genetic predisposition, although the genetic mechanisms are not clearly defined. This form of diabetes is often undiagnosed for many years. Nevertheless, patients are at increased risk of developing complications (ADA, 2012).

3. **Gestational diabetes**

Gestational diabetes is any degree of glucose intolerance with its onset or first diagnose during pregnancy. Approximately 7% of all pregnancies are accompanied by gestational diabetes (ADA, 2012). Although most cases resolve with delivery, women with gestational diabetes have 7-fold greater risk of developing T2DM later in life compared with those who have had a normoglycaemic pregnancy (Bellamy et al., 2009). Also offspring of diabetic mothers have increased risk for developing obesity and T2DM (Dabelea, 2007).

4. **Other specific types of diabetes**

Other types of diabetes can be due to genetic defects of the β-cells (maturity onset diabetes of the young, MODY), genetic defects in insulin action, diseases of the exocrine pancreas or disorders of the endocrine system. Diabetes can also be induced by drugs, chemicals or infections. Likewise, genetic syndromes (Down syndrome, Klinefelter syndrome, Turner syndrome, Wolfram syndrome etc.) and autoimmune diseases (stiff-man syndrome or systemic lupus erythematosus) are associated with an increased diabetes incidence (ADA, 2012).

1.3 Diagnostic criteria for diabetes

For many years, criteria for the diagnosis of diabetes were based on measurements of plasma glucose levels, either fasting or after an oral glucose tolerance test (OGGT). An Expert Committee on the Diagnosis and Classification of Diabetes Mellitus revised the diagnostic criteria (Nathan et al., 2009a) and since then the ADA recommends the following criteria (ADA, 2012):

- HbA1c ≥6.5%. The measurement should be performed using a method that is certified by the National Glycohaemoglobin Standardization Program (NGSP) and standardized to the Diabetes Control and Complication Trial (DCCT) reference assay.

OR

- Fasting plasma glucose ≥126 mg/dl (7.0 mmol/l), measured after no caloric intake for at least 8 hrs.

OR

- 2 hrs plasma glucose ≥200 mg/dl (11.1 mmol/l) during a 75 g OGTT, performed as described by the World Health Organization.

OR

- A random plasma glucose concentration ≥200 mg/dl (11.1 mmol/l) in patients with classic symptoms of hyperglycaemia or hyperglycaemic crisis.

In case of unequivocal hyperglycaemia, point 1-3 should be repeated to confirm the obtained result (ADA, 2012).

An intermediate group of individuals that does not meet the diagnostic criteria for diabetes but has increased fasting or postprandial plasma glucose levels is considered as having “pre-diabetes”.

The following criteria for the diagnosis of pre-diabetes are suggested by the ADA (ADA, 2012):

- Fasting plasma glucose: 100 mg/dl (5.6 mmol/l) - 125 mg/dl (6.9 mmol/l); individuals are defined as having impaired fasting glucose (IFG)
- 2 hrs postprandial glucose after an OGTT: 140 mg/dl (7.8 mmol/l) - 199 mg/dl (11.0 mmol/l); individuals are defined as having impaired glucose tolerance (IGT)
- HbA1c: 5.7 - 6.4%

Patients meeting these criteria are at higher risk for developing diabetes and cardiovascular diseases in the future (Tabák et al., 2012).

1.4 Glucose metabolism and pathogenesis of type 2 diabetes

In healthy individuals, plasma glucose levels remain in a narrow range, despite periods of fasting and feeding. Normal glucose concentrations (between 4 mmol/l (72 mg/dl) and 7 mmol/l (126 mg/dl)) are determined by homeostasis between glucose entering and glucose being removed from the circulation. The amount of glucose entering the blood stream is derived from intestinal absorption after food supply, glycogenolysis (breakdown of glycogen), and gluconeogenesis (formation of glucose from non-carbohydrate sources). Glycogenolysis and gluconeogenesis are partly under control of glucagon, secreted by α-cells of the pancreas. Glucose removal from the blood stream into mainly skeletal muscle and adipose tissue is triggered by insulin, secreted in the form of proinsulin by pancreatic β-cells (Nolan et al., 2011, Aronoff et al., 2004). Insulin is one of the most potent anabolic hormones in the human body. It promotes glucose uptake by stimulating the translocation of GLUT4 from intracellular sites to the plasma membrane and inhibits hepatic glucose production. Insulin also increases the rate of storage pathways such as lipogenesis (fatty acid synthesis and subsequent triglyceride synthesis), glycogen and protein synthesis, and it thereby promotes the storage of substrates in fat, liver and muscle. Furthermore, it inhibits lipolysis (breakdown of lipids), glycogenolysis and protein breakdown (Saltiel and Kahn, 2001).

Glucose is the most potent stimulus of insulin secretion. However, also amino acids, especially arginine, leucine, and lysine, and gut hormones released after a meal (glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP)) promote insulin secretion (Aronoff et al., 2004).

Type 2 diabetes is characterized by high blood glucose levels. Insulin resistance and pancreatic β-cell dysfunction are responsible for the onset of the disorder. They are usually present long time before hyperglycaemia and T2DM develop (Stumvoll et al., 2005). Figure 1 illustrates effects of decreased insulin secretion on glucose metabolism via interactions with brain, liver, muscle and adipocytes. Impaired insulin secretion affects signalling pathways in the hypothalamus, results in decreased suppression of hepatic glucose production and reduced efficiency of glucose uptake into the muscle. Furthermore, decreased insulin secretion increases lipolysis in adipocytes and thereby elevates levels of non-esterified fatty acids (NEFAs) (Kahn et al., 2006).

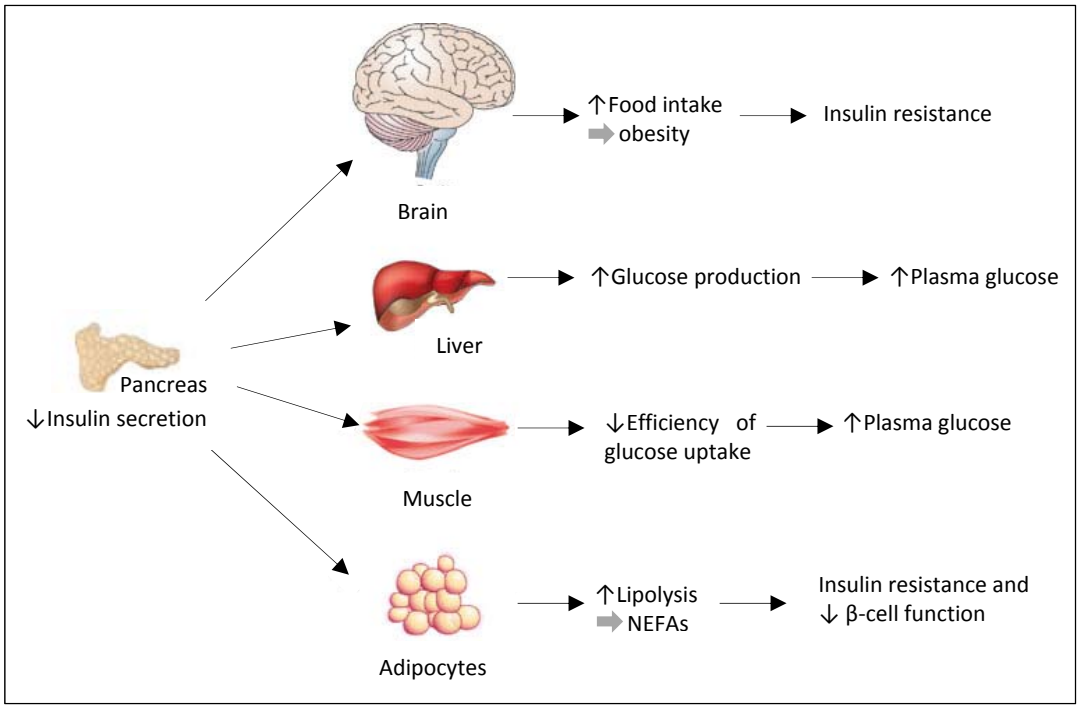


Figure 1. Impact of decreased insulin release by pancreatic β-cells on regulation of glucose levels.

NEFAs, non-esterified fatty acids

Reprinted with permission from *Nature (Kahn et al., 2006)*.

Insulin resistance results from an interaction of environmental and genetic factors, associated with overweight and obesity. Adipose tissue contributes to insulin resistance via endocrine and inflammatory pathways. It leads to an imbalance in hormone concentrations (increased leptin and reduced adiponectin), increased concentrations of cytokines (e.g. tumour necrosis factor α (TNF-α), interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1)) and increased release of NEFAs (Kahn et al., 2006, Schofield and Sutherland, 2012, Tahrani et al., 2011).

Pancreatic β-cells increase insulin release to compensate for decreased insulin sensitivity. If β-cells are unable to compensate for this imbalance, plasma glucose levels exceed the normal range (Nolan et al., 2011). β-cell dysfunction is suggested to be a result of decreased β-cell mass due to increased β-cell apoptosis (Butler et al., 2003), glucotoxicity, lipotoxicity (McGarry and Dobbins, 1999), or a combination of both, termed glucolipotoxicity (Kahn et al., 2006, Piñero-Piloña and Raskin, 2001). The mechanisms of glucotoxicity involve increased levels of reactive oxygen species (ROS) in β-cells, that damage the cell and enhance nuclear factor kappa B (NFκB) activity, which potentially induces β-cell apoptosis, while lipotoxicity is induced by chronically increased levels of fatty acids (Stumvoll et al., 2005).

It's worth mentioning that both, insulin resistance and β-cell dysfunction must be present to develop T2DM. Some chronically overweight or obese individuals do not develop T2DM, indicating that their β-cells successfully compensate for insulin resistance which underlines the role of genetic predisposition (Leahy, 2005, Nolan et al., 2011).

1.5 Diabetes and complications

Type 2 diabetes is a complex metabolic dysfunction and cannot be considered as a single disease, but rather as a group of metabolic disorders (Vigneri et al., 2009).

It is well recognized that T2DM is associated with increased risk for microvascular and macrovascular complications. Microvascular complications include retinopathy, nephropathy, neuropathy and can lead to blindness, renal failure, neuronal damage and foot ulcerations. Macrovascular complications affect arteries that supply heart, brain and lower extremities and result in increased risk for myocardial infarction, stroke and limb amputation (Brownlee, 2001, Fowler, 2011).

Furthermore, meta-analyses suggest associations between T2DM and several types of cancer. Findings strongly support a positive relationship for hepatic (El-Serag et al., 2006, Wang et al., 2012) and pancreatic cancer (Ben et al., 2011, Huxley et al., 2005), although the latter might reflect some degree of "reverse causality", since diabetes diagnosis might be associated with undiagnosed pancreatic cancer (Johnson et al., 2012). Increased risk of colorectal (Jiang et al., 2011, Larsson et al., 2005, Luo et al., 2012, Yuhara et al., 2011), bladder (Larsson et al., 2006), kidney cancer (Larsson and Wolk, 2011) and non-Hodgkin lymphoma (Castillo et al., 2012, Mitri et al., 2008) were observed in individuals with diabetes. Meta-analyses also suggest a positive relationship between diabetes and risk of endometrial cancer (Friberg et al., 2007) and breast

cancer (only in post-menopausal women) (Boyle et al., 2012). The association between diabetes and prostate cancer is discussed controversially. Interestingly, most studies report a negative association (Bansal et al., 2012, Kasper and Giovannucci, 2006, Zhang et al., 2010, Fowke et al., 2008). However, a two-year longitudinal study observed a positive association between HbA1c and levels of prostate-specific antigen (Ohwaki et al., 2011).

Several factors, like obesity, quality of metabolic control, medication, lifestyle, etc. have major impact on the onset of diabetes-associated. Especially for cancer, it is not clear yet if the association is direct or due to common predisposing risk factors such as age and obesity (Giovannucci et al., 2010). Mechanisms that are hypothesized as being responsible for increased cancer risk in T2DM patients include the effects of hyperglycaemia, insulin resistance and hyperinsulinaemia (Johnson et al., 2012).

1.5.1 Mechanisms of hyperglycaemia induced damage

Hyperglycaemia is suggested to be a strong determinant for micro- and macrovascular complications (Brownlee, 2001). In the case of cancer progression the role of hyperglycaemia is discussed controversially (Joshu et al., 2012, Miao Jonasson et al., 2012, Rapp et al., 2006). It has been suggested that hyperglycaemia facilitates neoplastic proliferation, since many cancers depend on glycolysis for energy generation (Polet and Feron, 2013).

Hyperglycaemia induces overproduction of ROS by the mitochondrial electron transport chain which activates four pathways that play a central role in the pathogenesis of diabetic complications: (1) increased polyol pathway flux, (2) increased advanced glycation end-product (AGE) formation, (3) activation of protein kinase C (PKC) isoforms, and (4) increased hexosamine pathway flux (Nishikawa et al., 2000, Brownlee, 2001).

Under normal physiological conditions interactions between mitochondrial protein complexes (complex I-IV) and electron donors (NADH and flavin adenine dinucleotide (FADH2)) generate a proton gradient across the mitochondrial membrane, which is used for ATP synthesis (Trumpower, 1990). Under hyperglycaemic conditions more glucose is converted to pyruvate during glycolysis, leading to increased generation of electron donors via the citric acid cycle. This leads to an increased proton gradient across the mitochondrial membrane, up to a critical threshold. At this point, electron transfer inside protein complex III is blocked leading to increased half-life of free-radical intermediates of coenzyme Q (ubiquinone), which reduce O_2 to superoxide (Brownlee, 2001, Brownlee, 2005, Giacco and Brownlee, 2010).

Excess superoxide production is suggested to induce DNA strand breaks which activate poly(ADP-ribosyl) polymerase (PARP), a DNA repair enzyme. Activated PARP splits nicotinamide adenine dinucleotide (NAD^+) into nicotinic acid and ADP-ribose (Figure 2). Poly(ADP-ribosyl) polymerase proceeds to make polymers of ADP-ribose (PAR) which accumulate on the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and consequently decrease its activity (Brownlee, 2005, Schreiber et al., 2006).

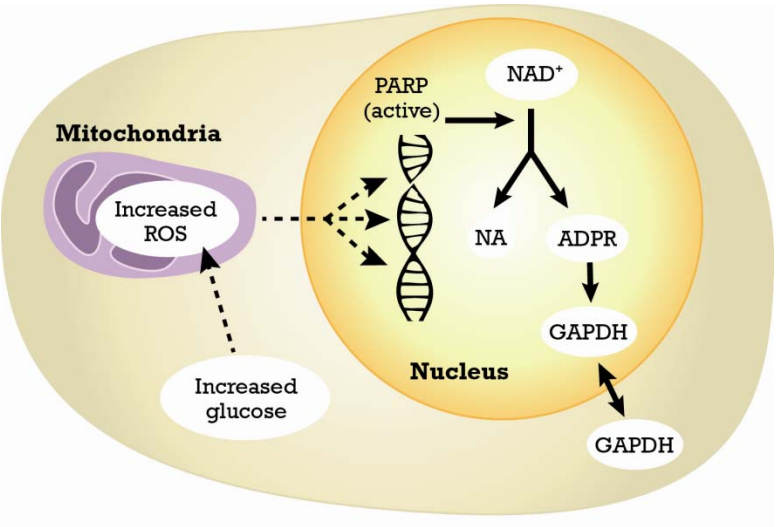


Figure 2. Excess ROS production causes DNA damage, activates PARP and modifies GAPDH by adding ADP-ribose polymers.

ADPR, ADP-ribose; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **NA**, nicotinic acid; **PARP**, poly(ADP-ribosyl) polymer; **ROS**, reactive oxygen species;

Modified after Brownlee, 2005

Decreased GADPH activity leads to increased levels of glycolytic intermediates that are upstream of GAPDH (Figure 3). Levels of the first glycolytic metabolite, glucose, are elevated in T2DM patients, causing increased flux through the polyol pathway. Furthermore, inhibition of GAPDH leads to increased levels of fructose-6-phosphate, which increase flux through the hexosamine pathway. Finally, high levels of the glycolytic metabolite glyceraldehyde 3-phosphate activates the PKC pathway and AGE pathway (Brownlee, 2001, Szabo, 2009).

1.5.1.1 Increased polyol pathway flux

Within the polyol pathway, carbonyl compounds, including glucose, are reduced to sorbitol by aldose reductase. Nicotinamide adenine dinucleotide phosphate (NADPH) is required as a

cofactor. Sorbitol can be oxidized to fructose by the enzyme sorbitol dehydrogenase using NAD^+ as co-factor (Brownlee, 2001). Both enzymes, aldose reductase and sorbitol dehydrogenase, are

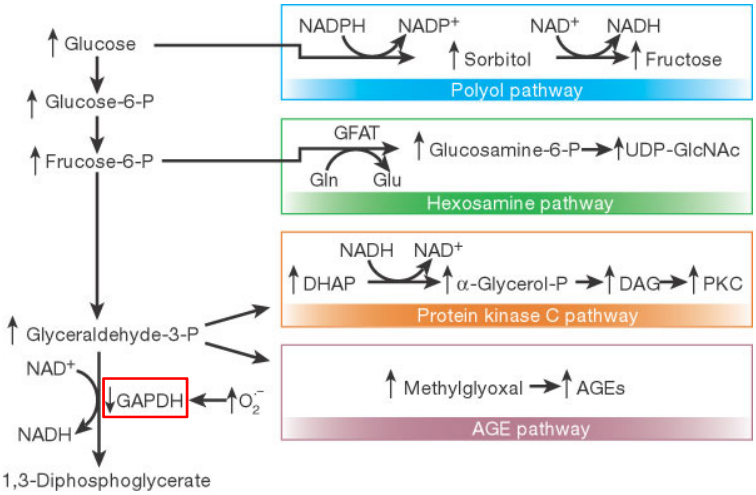


Figure 3. Pathways of glucose mediated vascular damage.

AGE, advanced glycated end product; **DAG**, diacylglycerol; **DHAP**, dihydroxyacetone phosphate; **GAPDH**, glyceraldehyde 3-phosphate dehydrogenase; **GFAT**, glutamine: fructose-6-phosphate amidotransferase, **Gln**; glutamine; **Glu**, glucose; **NAD^+/NADH**, oxidized/reduced nicotinamide adenine dinucleotide; **NADP+/NADPH**, oxidized/reduced nicotinamide adenine dinucleotide phosphate; **PKC**, protein kinase C; **UDP-GlcNAc**, uridine diphosphate-N-acetyl glucosamine;

Reprinted with permission from *Nature (Brownlee, 2001)*.

found in tissues prone to diabetic complications, like retina, lens, kidney, and nerve cells (Figueroa-Romero et al., 2008, King and Loeken, 2004). Aldose reductase has low affinity to glucose and therefore only small amounts are metabolized in healthy individuals via this pathway. In conditions of hyperglycaemia increased glucose levels result in increased conversion to sorbitol and its metabolite, fructose, that have been suggested to result in osmotic damage causing cataract formation and renal complications (Gugliucci, 2000). However, it has been proposed that sorbitol concentrations in vessels and nerves of diabetic individuals are too low to cause such damage (Brownlee, 2001), which might explain effectiveness of aldose reductase inhibitors in diabetic neuropathy (Oates, 2008).

Excess glucose flow through this pathway consumes NADPH. Nicotinamide adenine dinucleotide phosphate is required for regeneration of reduced glutathione (GSH), and therefore increased polyol pathway flux could induce oxidative stress (Brownlee, 2001). Furthermore, NADPH is needed for the synthesis of nitric oxide (NO), which is important for vasodilation of blood vessels. Low NO synthesis might lead to vasoconstriction and poor blood supply (Gabbay, 1973).

1.5.1.2 Increased flux through the hexosamine pathway

During glycolysis, glucose is converted to fructose-6-phosphate (Fruc-6-P). The entry of Fruc-6-P into the hexosamine pathway is catalysed by the enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT). As illustrated in Figure 3 Fruc-6-P is converted to glucosamine-6-phosphate and then to uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc). Uridine diphosphate-N-acetylglucosamine is the major end product of the hexosamine pathway and regulates glucose entry into the pathway since it is the allosteric feedback inhibitor of GFAT. Furthermore, UDP-GlcNAc is the obligatory substrate of *O*-GlcNAc transferase (OGT), which catalyses the attachment of *O*-GlcNAc on serine and threonine residues of cytoplasmic and nuclear proteins (Brownlee, 2001, Marshall et al., 1991).

Numerous proteins, including kinases, phosphatases, transcription factors, metabolic enzymes, chaperons and cytoskeletal proteins can be modified by *O*-GlcNAc (Fülöp et al., 2007), leading to changes in both gene expression and protein function. Modification of the transcription factor Sp1 (Specific Protein 1) by UDP-GlcNAc causes activation of the plasminogen activator inhibitor-1 (PAI-1) promoter and stimulates expression of transforming growth factor-β1 (TGF-β1) (Brownlee, 2001). Higher PAI-1 levels are discussed to be involved in diabetic neuropathy (Hafer-Macko et al., 2007), while TGF-β1 is suggested to be involved in diabetic nephropathy (Kanwar et al., 2008). Also endothelial nitric oxide synthase (eNOS) activity can be inhibited via increased hexosamine pathway flux, which is relevant to atherosclerotic risk (Du et al., 2001).

1.5.1.3 Activation of protein kinase C isoforms

Hyperglycaemia can induce vascular dysfunction via formation of diacylglycerol (DAG) and subsequent activation of PKC isoforms. Activation can also be indirectly mediated by hyperglycaemia, through increased polyol pathway flux or ligation of AGE receptors (Keogh et al., 1997, Koya and King, 1998).

Brownlee extensively reviewed the multiple effects of PKC activation gained from animal and cell culture studies (Brownlee, 2001). Protein kinase C activation depresses NO production, increases endothelin-1 activity and affects expression of eNOS leading to blood flow abnormalities. It increases vascular permeability by inducing the expression of vascular endothelial growth factor (VEGF) in smooth muscle cells, mediates capillary and vascular occlusion and affects pro-inflammatory gene expression via activation of NFκB. In addition, hyperglycaemia induced activation of PKC activates NAD(P)H oxidases causing increased levels of ROS (Brownlee, 2001).

1.5.1.4 Increased intracellular formation of advanced glycation end-products

Advanced glycation end-products are formed by a non-enzymatic reaction of the carbonyl group of reducing sugars with a free amino group of proteins, lipids, or nucleic acids. The precursors of AGEs are reactive dicarbonyls, like glyoxal (formed via oxidation of glucose), 3-deoxyglucosone (formed via degradation of Amadori products) and methylglyoxal (Brownlee, 2001). These intermediate products are reversible, while AGEs are nearly irreversible (Schmidt et al., 1999).

Advanced glycation end-product formation leads to cell damage via three general mechanisms. First, AGE precursors can modify intracellular proteins and alter their function. Second, AGE precursors can diffuse out of the cell and modify extracellular matrix proteins, which leads to abnormal interaction with other matrix components or matrix receptors (Brownlee, 2001). The formation of AGEs on vascular wall and myocardial collagen causes cross-linking of collagen molecules to each other. This leads to decreased elasticity in large vessels and increased fluid filtration (Aronson, 2003). Third, plasma proteins modified by AGE precursors can interact with AGE receptors (RAGE) on macrophages, mesangial-and endothelial cells. This induces the expression of cytokines and growth factors by macrophages and mesangial cells (e.g. interleukin-1, insulin-like growth factor-I, TNF-α) and expression of pro-coagulatory and pro-inflammatory molecules by endothelial cells (thrombomodulin, tissue factor and cell adhesion molecule VCAM-1) (Brownlee, 2001, Giacco and Brownlee, 2010). The interaction between AGEs and RAGE also induces the production of ROS (Coughlan et al., 2009).

1.5.2 Mechanisms of hyperinsulinaemia induced damage

Insulin resistance and consequent hyperinsulinaemia are usually present several years before the diagnosis of T2DM. Hyperinsulinaemia is discussed as a possible factor increasing cancer risk due to the mitogenic effect of insulin. The mechanisms behind insulin's mitogenic effects are manifold and complex (Cohen and LeRoith, 2012). Activation of the insulin receptor (IR) stimulates intracellular signalling cascades which have mitogenic and antiapoptotic potential. However, these effects are suggested to occur only at supraphysiological insulin concentrations (Roberts et al., 2010).

Insulin's proliferative effects are suggested to be related to increased production and also biological activity of **insulin-like growth factor-1** (IGF-1), a peptide hormone whose molecular structure is very similar to that of insulin. Increased production of IGF-1 is due to high growth hormone receptor (GHR) levels in individuals with T2DM. Activation of GHR through growth hormone (GH) results in increased IGF-1 secretion (Baxter and Turtle, 1978, Calle and Kaaks, 2004,

Kaaks and Lukanova, 2001). Furthermore, biological activity of IGF-1 is increased due to reduced hepatic production of insulin-like growth factor binding protein (IGFBP) 1 and 2. Since IGFBP-1 and 2 usually bind IGF-1, reduced concentrations of these two proteins lead to increased levels of free and bioactive IGF-1 (Firth and Baxter, 2002).

Insulin-like growth factor-1 activates the IGF-1 receptor (IGF-1R), which induces several biological actions, like cell proliferation, differentiation, and inhibition of apoptosis, which may favour tumour growth (Khandwala et al., 2000). Furthermore IGF-1 plays an important role in tumour invasion, since activation of IGF-1R kinase leads to a loss of endothelial coherence and promotes cell migration (Chan and Lee, 2008, Roberts et al., 2010). Also insulin can activate the IGF-1R and thereby exert mitogenic effects. However, due to the low affinity of insulin to the IGF-1R its relevance for carcinogenesis is not clear yet (Mynarcik et al., 1997) and mitogenic effects in vitro were only observed at very high concentrations (Sandhu et al., 2002).

Furthermore, the **insulin receptor** itself has impact on carcinogenesis. It can be expressed in two different isoforms, IR-A and IR-B, whereby IR-A is mainly expressed during foetal development. Also cancer cells show significant levels of IR-A. Insulin receptor-B is expressed in target tissues for insulin action, like liver, muscle, adipose tissue, and kidney (Belfiore and Malaguarnera, 2011) and is responsible for mediating metabolic effects, like regulating glucose uptake. Insulin receptor-A is suggested to mediate non-metabolic effects and to stimulate insulin-mediated mitogenesis (Benecke et al., 1992). Insulin receptor-A compared to IR-B has increased affinity for IGFs, especially for IGF-2 and thereby leads to cell growth, proliferation, and survival (Belfiore and Malaguarnera, 2011). In individuals with T2DM a high IR-A:IR-B ratio was observed (Denley et al., 2003). However, the role of increased IR-A levels is still unclear, but might contribute to increased cancer risk under hyperinsulinaemic conditions.

Besides that, cancer cells show a high IR content. An overexpression of IRs was observed in human breast, ovarian and colon cancer (Arcidiacono et al., 2012, Papa et al., 1990) which may result in oncogenic effects via abnormal stimulation of signalling cascades, affecting cell metabolism, cell survival, proliferation, and migration (Belfiore and Malaguarnera, 2011, Law et al., 2008). Furthermore, in cancer cells expressing both IR and IGF-1R, hybrid receptors containing an IR hemireceptor and an IGF-1R hemireceptor can be formed (Häring et al., 1994). These hybrid receptors bind IGF-1 with higher affinity than insulin, and thereby mediate mitogenic effects (Soos et al., 1993).

Hyperinsulinaemia also has effects on bioavailability of other hormones, especially **sex hormones**. Insulin reduces the hepatic synthesis of sex hormone binding globulin (SHBG), leading to

increased bioavailability of estradiol (in men and women) and testosterone (in women but not in men) (Calle and Kaaks, 2004). The relation between insulin and sex hormone bioavailability is further influenced by the occurrence of obesity in individuals with hyperinsulinaemia. In adipose tissue the expression of enzymes promoting the conversion of androgenic precursors to estradiol and testosterone is increased. The combination of increased sex hormone concentrations and reduced SHBG levels increases bioavailability of sex hormones. Binding to their receptors mediates cell proliferation and inhibits apoptosis (Levin, 2002). However, effects of sex hormones depend strongly on the tissue type, where they bind to their receptor. Proliferative effects were observed in endometrial (Kaaks et al., 2002) and breast cancer tissues (in postmenopausal women) (Key et al., 2003).

Hyperinsulinaemia is also discussed as being associated with polycystic ovary syndrome in individuals who are genetically susceptible, since insulin can increase ovarian androgen concentrations (Nestler, 1997).

1.5.3 The Impact of obesity and inflammation

More than 80% of T2DM patients are overweight (Vigneri et al., 2009). Adipose tissue plays a central role in energy metabolism, lipid metabolism and is considered as an “endocrine organ”, producing a variety of metabolically active compounds, known as adipocytokines. Adiponectin and leptin are the most studied adipocytokines, and provide an important link between obesity, diabetes and associated complications (Tilg and Moschen, 2006). Adipose tissue can contain a considerable number of immune cells, including macrophages and T lymphocytes. The number of macrophages in white adipose tissue make a considerable contribution to the production of inflammatory cytokines (Antuna-Puente et al., 2008), and correlate with obesity (Tilg and Moschen, 2006).

Therefore, obesity is associated with abnormal cytokine levels, increased concentrations of C-reactive protein (CRP) and disturbed adipokine levels (increased leptin, visfatin, resistin; decreased adiponectin) which contribute to the observed low-grade inflammatory conditions and play an important role in the pathogenesis of insulin resistance (Forte et al., 2012, Tilg and Moschen, 2006).

There is scientific evidence that the interplay between obesity and low-grade inflammation substantially contributes to the onset of micro- and macrovascular (Joussen et al., 2004, Mantovani et al., 2008, Satoh et al., 2003) complications. The role of inflammation in cancer development is not fully understood. It’s suggested that inflammation can initiate or promote

malignant transformation (Yu et al., 2009) probably via an increase in cell turnover, which might together with persistent DNA damage, end in cellular transformation (Ames et al., 1995, Dranoff, 2004). Furthermore, abnormal cytokine signalling might lead to altered cell growth, differentiation and apoptosis (Dranoff, 2004). Receptors for IL-6 and TNF-α are often expressed on tumour cells and through binding they can increase tumour growth and migration (Pollard, 2004). Tumour necrosis factor-α has been found to induce DNA damage and inhibit DNA repair (Jaiswal et al., 2000) via the formation of NO (Nussler et al., 1993). In addition, inflammation and cancer are suggested as being linked through the activation of transcription factors (NFκB, signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1α (HIFIα)) which lead to an up regulation of genes involved in cell proliferation (Yu et al., 2009), influence tumour-cell migration, invasion and metastasis (Mantovani et al., 2008).

Tumour necrosis factor-α suppresses the transcription of adiponectin (Maeda et al., 2002), an adipocytokine with anti-atherogenic and anti-inflammatory properties, that is negatively associated with obesity and insulin resistance (Forte et al., 2012). Regarding tumour biology, adiponectin is suggested as being antiproliferative, proapoptotic, and antiangiogenic (Roberts et al., 2010). Leptin, on the other hand, is positively correlated to obesity and acts as proinflammatory agent, via the stimulation of STAT3, resulting in increased levels of TNF-α and IL-6 (Tilg and Moschen, 2006). In addition, leptin has proangiogenic properties and suppresses apoptosis (Artwohl et al., 2002).

1.5.4 The Impact of medication on cancer risk

The main aim in diabetes treatment is establishing a stable glucose metabolism to prevent diabetes associated complications. Several factors, like patient characteristics, comorbidities, and known acute and chronic adverse effects of treatment, have considerable impact on the choice of pharmacological therapies (Giovannucci et al., 2010). In T2DM patients, oral anti-diabetic drugs are usually used early in the course of the disease, while insulin treatment is mostly used if patients that are insulin deficient do not respond to oral anti-diabetic agents (Johnson et al., 2012, Nolan et al., 2011). However, insulin is also used in newly diagnosed T2DM patients for short time since it is suggested to have better effects on β-cell function and to lead to prolonged glycemic remission compared to oral anti-diabetic agents (Weng et al., 2008).

The therapeutic choice regarding oral anti-diabetic agents for the management of hyperglycaemia or insulin resistance are manifold. Different drugs act at different tissue sites and lead to improved glucose control via various mechanisms (Figure 4).

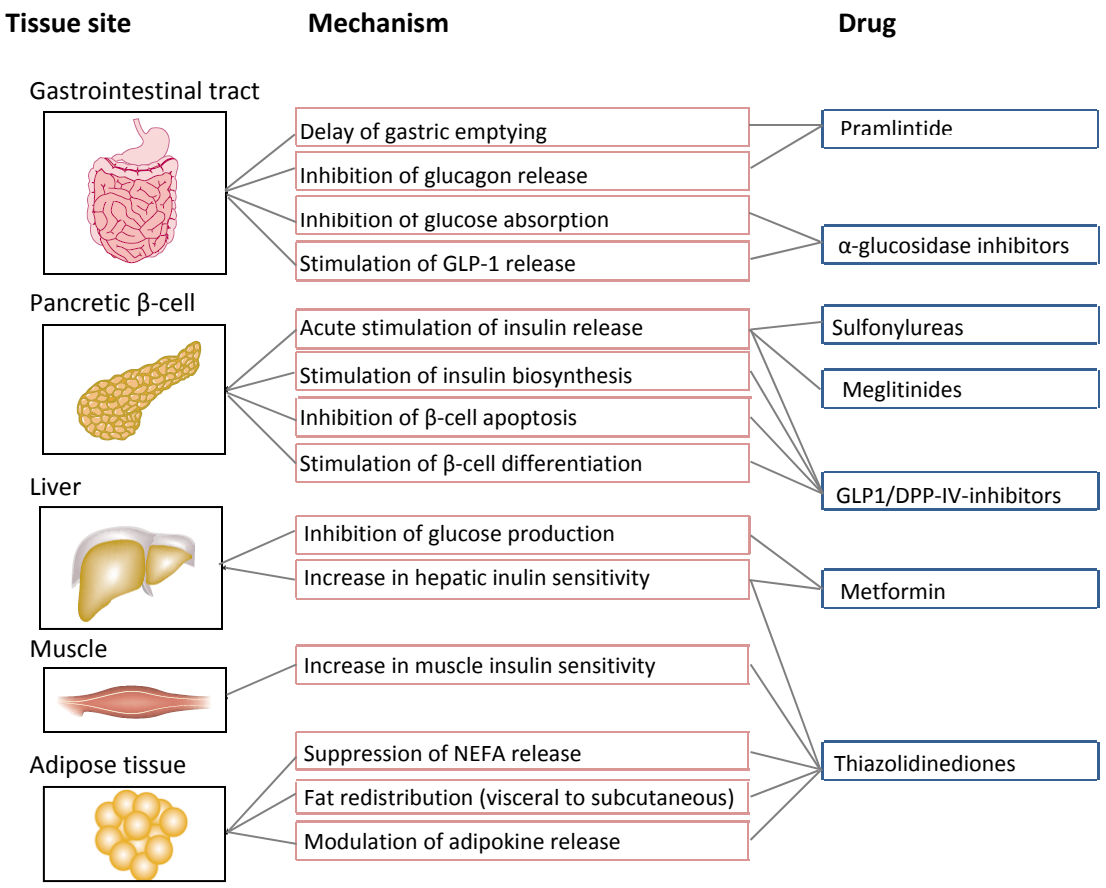


Figure 4. Therapeutic choices for the treatment of hyperglycaemia.

DPP-IV-inhibitor, dipeptidyl peptidase IV-inhibitor; **GLP-1**, glucagon-like peptide-1; **NEFA**, non-esterified fatty acid;

Reprinted with permission from *The Lancet (Stumvoll et al., 2005)*

Pramlintide and α-glucosidase inhibitors act in the gastrointestinal tract, while sulfonylureas, meglitindes, GLP-1/ dipeptidyl peptidase IV (DPP-IV) inhibitors act in the pancreas and enhance insulin secretion. Metformin as well as thiazolidinediones (TZDs) lead to increased insulin sensitivity in the liver, the latter additionally increases muscle insulin sensitivity (Chowdhury, 2010, Stumvoll et al., 2005).

Regarding cancer risk, it has been suggested that drugs which reduce insulin levels may reduce cancer risk, while exogenous insulin or drugs which increase endogenous insulin levels may increase cancer risk (Johnson et al., 2012, Chowdhury, 2010).

Metformin is suggested as initial treatment (in the absence of specific contraindications, like renal insufficiency) in patients with T2DM (Nathan et al., 2009b). It decreases circulating glucose levels by reducing hepatic glucose production and by increasing hepatic insulin sensitivity (Stumvoll et

al., 2005). Within cell culture studies with endometrial (Cantrell et al., 2010) or breast cancer cells (Alimova et al., 2009), metformin was found to inhibit cancer cell growth and proliferation and to cause cell cycle arrest, respectively. In an observational cohort study with T2DM patients, cancer risk was compared between metformin users and those that had never received metformin. The results suggested reduced cancer risk in patients treated with metformin (Libby et al., 2009). These findings are supported by a prospective study investigating the association between the use of metformin and cancer mortality, reporting lower cancer mortality in T2DM patients using metformin compared to non-users (Landman et al., 2010). Regarding prostate and breast cancer, treatment with metformin was associated with reduced cancer risk (Bodmer et al., 2010, Wright and Stanford, 2009). The positive effect of metformin is suggested to be due to the activation of AMP-activated protein kinase (AMPK), which interferes with cancer cell metabolism, rather than affecting insulin levels (Zakikhani et al., 2006).

However, the positive results from observational trials could not be confirmed within a meta-analysis of randomized clinical trials investigating cancer outcomes and all-cause mortality in adults treated with metformin: no effect of metformin on cancer outcomes was observed (Stevens et al., 2012). The heterogeneity of diabetic conditions and the complexity of cancer initiation and progression make it hard to draw firm conclusions.

Thiazolidinediones primarily act in the adipose tissue via activation of peroxisome proliferator-activated receptor gamma (PPAR γ), they increase subcutaneous adipogenesis, reduce the release of FFA and increase hepatic and muscle insulin sensitivity (Tahrani et al., 2011). TZDs improve dyslipidaemia, inflammatory condition and increase adiponectin concentrations (Stumvoll et al., 2005). The influence of TZDs on cancer incidence is discussed controversially: they were associated with reduced lung cancer risk, while no effect on colorectal and prostate cancer was observed (Govindarajan et al., 2007). In contrast Ramos-Nino et al. suggested a positive association between TZD use and cancer (Ramos-Nino et al., 2007) and Koro et al. found no association between TZDs and risk of colon, prostate and breast cancer (Koro et al., 2007). A meta-analysis of 80 trials investigating the effect of rosiglitazone on cancer incidence concluded that rosiglitazone was safe in terms of cancer incidence (Monami et al., 2008).

Another type of drug used to control blood glucose levels are **sulfonylureas** and rapid-acting glinides which increase insulin secretion (Giovannucci et al., 2010). Within observational studies, the use of sulfonylureas was associated with a higher risk for developing of solid cancers (Currie et al., 2009) and higher cancer-related mortality (Bowker et al., 2006, Monami et al., 2009) compared to patients treated with metformin. Giovannucci et al. suggested that based on the

above mentioned trials no firm conclusions can be drawn since it remains unknown whether results reflect increased cancer risk in patients treated with sulfonylureas, or reduced risk in metformin users (Giovannucci et al., 2010).

Incretin-mimetics and DPP-IV inhibitors belong to the group of **incretin-based drugs**. Incretin-mimetics mimic the action of GLP-1 which is produced in the gut and improve glucose-dependent insulin release, decrease glucagon secretion and delay gastric emptying. Dipeptidyl peptidase IV inhibitors block the enzyme DPP-IV which inactivates GLP-1 and thereby leads to increased GLP-1 levels (Giovannucci et al., 2010, Nolan et al., 2011). Incretin based agents are rather new drugs and therefore long-term studies investigating their effect on cancer risk are not available. In rodents, GLP-1 receptor agonists were associated with thyroid cancer while this effect was not observed in monkeys. It is hypothesized, that the number of GLP-1 receptors on thyroid glands, which is by nature markedly lower in monkeys, is responsible for the different effect on cancer development (Knudsen et al., 2010). Elashoff et al. examined the US Food and Drug Administration's database and reported a positive association between GLP-1 based therapy and risk of pancreatitis and pancreatic cancer (Elashoff et al., 2011).

Insulin and insulin analogs are mainly used in T2DM patients with progressive loss of β-cell function. Circulating insulin levels after subcutaneous insulin injection usually exceed endogenous insulin levels. Due to the mitogenic properties of insulin, hyperinsulinaemia caused by insulin injection was suggested as being associated with increased cancer risk (Giovannucci et al., 2010).

In a retrospective, observational study Hemkens et al. compared the risk of malignant neoplasms and mortality between T2DM patients treated with either human insulin, apart, lispro (both are short acting insulin analogs) or glargine (long-acting insulin analog). For all insulin types a positive association between cancer incidence and insulin dose was observed. Furthermore, it was suggested that insulin glargine might have greater mitogenic activity, probably due to its higher affinity to the IGF-receptor, than human insulin (Hemkens et al., 2009). Based on these results retrospective studies analysing nationwide clinical databases or diabetes registers were performed addressing the issue of cancer risk in patients treated with insulin glargine compared to other types of insulin. Results from two studies suggested a positive association between the use of insulin glargine alone and incidence of all cancers (Colhoun, 2009) or breast cancer incidence (Jonasson et al., 2009). Currie et al. suggested no effect of insulin glargine on cancer risk (Currie et al., 2009). However, the above mentioned observational studies have some limitations since they were not fully controlled for confounding factors such as diabetes duration, body weight, quality of glucose control and use of other therapies (Chowdhury, 2010). In a recently

published large randomized control trial with a median follow-up of 6.2 years insulin glargine had a neutral effect on cancer incidence, mortality or cancer at specific sites (Gerstein et al., 2012).
To summarize, there is no evidence of an overall increase in cancer development in patients treated with insulin glargine. To draw firm conclusions about the impact of diabetes medication on cancer risk further studies considering confounders such as age, BMI, gender, changes in drug dosage and/or type during the course of the disease, duration of diabetes and quality of glycemic control are needed (Smith and Gale, 2009, Vigneri et al., 2009).

1.6 The role of diet in the management of diabetes

Diet is a central component of diabetes management (Nolan et al., 2011). Modification of dietary habits helps to control predisposing factors and thereby reduces the risk for diabetes complications. Based on a position statement of the ADA, nutrition recommendations for T2DM patients should be based on (1) the achievement or maintenance of optimal blood glucose levels, a lipid and lipoprotein profile that reduces the risk of vascular complications, optimal blood pressure levels and (2) the modification of nutrient intake and lifestyle to avoid the onset of chronic complications (ADA, 2008). The ADA does not give specific recommendations for macronutrient intake for T2DM patients. They support the idea that recommendations for the general population are also applicable for T2DM patient (Wheeler et al., 2012). The ADA underlines the importance of appropriate energy intake, increased physical activity to achieve or maintain optimum body weight, minimized trans FA consumption, cholesterol intake <200 mg/day, and reduced intake of saturated fatty acids (SFA) (<7% of total energy) (ADA, 2008).
Scientific evidence suggests that increased SFA intake is, among other factors, responsible for the increase in diabetes prevalence (Kolb and Mandrup-Poulsen, 2010). Furthermore, an inverse association between the risk of T2DM and the replacement of SFA by polyunsaturated fatty acids (PUFA) (Meyer et al., 2001), or increased PUFA intake alone (Salmerón et al., 2001) was observed.
Regarding diabetes complications, Brownlee suggested that hyperglycaemia derived oxidative stress is the key initiator for especially microvascular complications (Brownlee, 2001). However, due to lack of convincing results of antioxidant supplementation trials, daily vitamin or mineral supplements are currently not recommended by ADA, unless deficiencies are present. Vitamin and mineral requirements should rather be acquired via natural products and a balanced diet (ADA, 2008). The excess production of free radicals should be reduced via proper glycemic control, rather than scavenging already produced radicals (Ceriello and Testa, 2009).

1.7 DNA damage in diabetic individuals and its biomarkers

Hyperglycaemia, insulin resistance and hyperinsulinaemia, which are present in T2DM individuals, play an important role in mediating secondary complications. Especially hyperglycaemia induces increased ROS production (Brownlee, 2001), which can lead to oxidation of biomolecules such as proteins, lipids and nucleic acids (Finkel and Holbrook, 2000).

Markers of intracellular oxidative stress are the DNA oxidation marker 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and the RNA oxidation marker 8-oxo-7,8-dihydroguanosine (8-oxoGuo), which can be assessed in urine. It is important to recognize that in steady state the amount of 8-oxodG and 8-oxoGuo in urine reflect the average rate of oxidative damage in the whole body (Poulsen, 2005, Poulsen et al., 2012). Broedbaek et al. reviewed the role of 8-oxodG as a biomarker for therapy control and risk stratification in T2DM patients and summarized that there is evidence of consistent associations between T2DM, its complications and 8-oxodG excretions (Broedbaek et al., 2011b). The authors also suggest, due to the observed associations between 8-oxodG and cancer (Miyake et al., 2004, Tagesson et al., 1995, Thanan et al., 2008), that 8-oxodG levels in urine could reflect important pathogenetic mechanisms in cancer development in diabetic individuals (Broedbaek et al., 2011b). The RNA oxidation product, 8-oxoGuo, was suggested as an independent predictor of all-cause and diabetes related mortality in newly diagnosed T2DM patients (Broedbaek et al., 2012).

An alternative method for detecting DNA damage is the single cell gel electrophoresis (SCGE) assay. It allows the detection of DNA strand breaks and alkali-labile sites. The latter consist of apurinic and apyrimidinic sites and are formed after the loss of a damaged base, occur as intermediates during base excision repair or arise spontaneously due to altered chemical stability resulting from changes in bases or sugars (Collins et al., 2008). The SCGE assay can be combined with lesion-specific endonucleases which allow the detection of oxidized bases and increase the specificity of the assay. Endonuclease III converts oxidized pyrimidines, including thymine glycol and uracil glycol to strand breaks. Formamidopyrimidine DNA glycosylase (FPG) recognizes the oxidized purine 8-oxo-7,8-dihydroguanine (8-oxoGua), ring-opened purines, or formamido-pyrimidines (Azqueta et al., 2009). 8-oxo-7,8-dihydroguanine is one of the most abundant oxidized bases and it has been suggested as being involved in tumour initiation, promotion and progression (Cooke et al., 2003).

Another biomarker which is used for the assessment of genomic stability and cancer risk is the micronucleus (MN) frequency in lymphocytes measured by the cytokinesis-block micronucleus

(CBMN) assay (Bonassi et al., 2007, Murgia et al., 2008). The assessment of DNA damage with the CBMN assay is based on the scoring of DNA damage events in once-divided binucleated cells (Fenech, 2007). It enables the detection of MNi, nucleoplasmic bridges (NPBs) and nuclear buds (Nbuds). Micronuclei originate from whole chromosomes or chromosome fragments that failed to be incorporated in the main nucleus during mitosis. In contrast, NPBs are a biomarker of DNA misrepair and/or telomere end-fusion. Nuclear buds result from gene amplification and are a biomarker of elimination of amplified DNA and/or DNA repair complexes. Furthermore cytotoxicity can be measured via the assessment of necrosis and apoptosis (Fenech, 2007).

Micronucleus frequency can also be evaluated in exfoliated buccal cells with the buccal micronucleus cytome (BMCyt) assay (Thomas et al., 2009). In contrast to the measurement of MNi in lymphocytes, the BMCyt assay is minimally invasive and cells are directly applied on microscope slides after several washing steps. The BMCyt assay allows the assessment of DNA damage (MNi and Nbuds), cytokinetic defects (binucleated cells) and cell death (condensed chromatin, karyorrhexis, pyknotic and karyolitic cells).

In diabetic individuals higher frequencies of MNi in lymphocytes (Martínez-Pérez et al., 2007, Shettigar et al., 2012) and buccal cells (Lazalde-Ramos et al., 2012, Zúñiga-González et al., 2007) compared to healthy participants were observed. Furthermore, several nutrients (calcium, folate, nicotinic acid, vitamin E, retinol and β-carotene) were identified as being involved in maintaining or even improving genome stability (Fenech et al., 2005, Thomas et al., 2011). Thomas et al. reviewed the effect of dietary interventions on MN frequency in lymphocytes and buccal cells and concluded that supplementations with antioxidants and vitamins may reduce genome damage, whereas multiple antioxidants appear more efficacious than single antioxidants (Thomas et al., 2011). The effect of natural foods on chromosomal stability in diabetic individuals has not been investigated.

To conclude, oxidative DNA damage and genomic instability can cause mutations (Cooke et al., 2003) and are involved in the aetiology of diseases, such as cancer (Bonassi et al., 2007, Stratton et al., 2009) and CVD (Botto et al., 2001, Collins et al., 1998, Mercer et al., 2010). Diabetes is suggested as being associated with increased DNA damage (Arif et al., 2010, Lodovici et al., 2008) and chromosomal stability (Martínez-Pérez et al., 2007, Shettigar et al., 2012) and at the same time accompanied by increased risk for CVDs (Hu et al., 2001) and cancer (Vigneri et al., 2009). This highlights the need of further research on the effect of diet, which is a cornerstone in the treatment of T2DM, on biomarkers associated with secondary complications.

2 Objectives of the Thesis

- To compare levels of oxidative damage to DNA between diabetic and non-diabetic individuals
- To compare levels of chromosomal damage in lymphocytes and buccal cells between diabetic and non-diabetic individuals
- To evaluate the effect of an intervention (either 25 ml PUFA-rich walnut oil replacing SFA and 300 g of vegetables per day for 8 weeks or “only information”) on vitamins, antioxidants and fatty acids
- To assess the impact of the intervention on quality of glycemic control
- To investigate the potential of the dietary intervention to improve oxidative damage to DNA
- To measure the impact of the dietary intervention on levels of chromosomal damage in lymphocytes and buccal cells

3 Further Objectives of the Study

The present thesis aimed to compare DNA and chromosomal stability between diabetic and non-diabetic individuals and to assess the impact of a dietary intervention on these biomarkers. To get a better insight into mechanisms behind changes in biomarkers used in the present study, further investigations are warrant and ongoing in our lab. However, they were not the primary objective of the present dissertation.

Further objectives (not part of this dissertation) are:

- Assessment of DNA repair (base excision repair). The net amount of oxidative damage to DNA reflects the balance between DNA repair and antioxidative defence mechanisms that are capable of decreasing oxidative damage. Therefore, the assessment of DNA repair processes will provide further mechanistic insight.
- Performance of metabolomics analysis, which have been successfully used to understand complex interactions between diet and human physiological status. The use of metabolomics within this project will provide further insight regarding the metabolic effects of the present intervention. In addition it will be useful to study if there is any association between the profile of metabolites and the rate of DNA oxidation and chromosomal damage.
- Assessment of the effect of the intervention on lipid metabolism which is very important due to the increased risk for CVDs in diabetic individuals. The analysis of LDL-subfractions will give further insights into atherogenic processes.

4 Subjects and Methods

4.1 Study design and participants

The study was a randomized, parallel intervention study approved by the Ethical Committee of the City of Vienna (EK09-218-VK_NZ). Patients with established T2DM (36 treated with insulin (16 male, 20 female), 40 treated with oral antidiabetic medication (18 male, 22 female)) were recruited from a local diabetes clinic (Diabetes outpatient Clinic, Health Centre South, Vienna, Austria). Non-Diabetic individuals (n=21, 6 male, 15 female) were partners of the diabetic subjects.

All participants (diabetic and non-diabetic individuals) received information about the beneficial effects of a healthy diet with special focus on the importance of fat quality and the role of vegetables in a balanced diet. Participants were randomly assigned to the "intervention" or "information only" group. Subjects of the "information only" group only received the above mentioned information, while subjects of the "intervention" group additionally received 300 g of vegetables and 25 ml of PUFA rich walnut-oil per day. The intervention period lasted 8 weeks, followed by a period of 8 weeks in which no intervention foods were provided. Blood samples were taken before the intervention, after 4, 8 (end of intervention period) and 16 weeks (Figure 5).

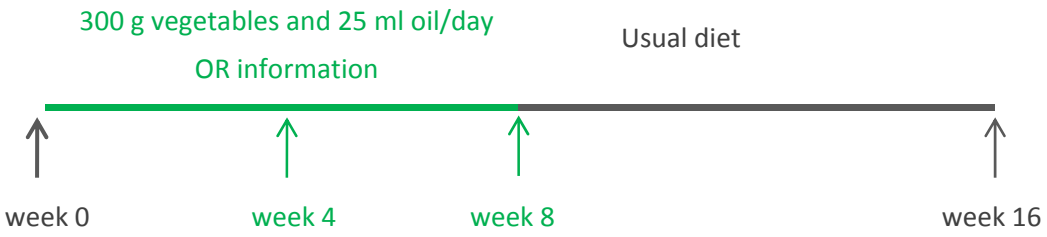


Figure 5. Study design

Participants were instructed to use the plant oil as replacement for SFA. A reference "cup" and a booklet with recipes, instructions for replacement of SFA and usage of the plant oil (oil was not allowed to be heated up, but added to warm foods) was provided to the participants. A variety of frozen vegetables (Table 1) was given to the subjects every 2 weeks. Composition of the plant oil is shown in Table 2.

Table 1. Intervention vegetables (amount for 14 days)

Amount (g)	Vegetables
225	Strained spinach
400	Leave spinach
300	Green beans
300	Broccoli
300	Brussels sprouts
300	Soybeans
300	Peas
300	Carrots[1]
400	Romaine lettuce with peas
300	Vegetable mix I (broccoli, cauliflower)
300	Vegetable mix II (broccoli, carrot yellow and orange)
300	Vegetable mix III (carrot, potato, kohlrabi, leek, pea)
300	Vegetable mix IV (pea, carrot, corn)
300	Vegetable mix V (carrot, potato, broccoli, green bean, cauliflower)
800	Roasted vegetables I (carrot, broccoli, cauliflower, pole beans, zucchini)[2]
800	Roasted vegetables II (carrot, Brussels sprouts, kohlrabi, pole beans)[2]
400	Ready meal: spinach with potatoes[3]

[1] was given to subjects additionally, in case of any digestive discomfort

[2] Subjects got roasted vegetables I for the first 14 days and roasted vegetables II for the second 14 days

[3] Contains 230 g of spinach

Table 2. Composition of the plant oil

	% of total fatty acid
C16:0	7.28 ± 0.09
C18:0	2.06 ± 0.07
C18:1n9c	16.4 ± 0.99
C18:1n7c	0.95 ± 0.03
C18:2n6c	61.8 ± 1.00
C18:3n3	11.5 ± 0.43
	mg / 100 g
γ-Tocopherol	33.0 ± 1.82
α-Tocopherol	2.67 ± 0.14
Campesterol	6.20 ± 0.45
Campestanol	1.00 ± 0.00
Stigmasterol	1.00 ± 0.00
Sitosterol	116 ± 5.00
Sitostanol	1.20 ± 0.45
Δ 5-avenasterol	11.6 ± 0.89
Cycloartenol + Δ 7-stigmastenol	38.0 ± 5.70
Minor sterols	7.80 ± 0.84
Total plant sterols	183 ± 2.10

Recruitment of participants was based on the following **inclusion criteria**:

- Men and women with T2DM
    - 40 to 80 years of age
    - treatment with oral antidiabetic agents (metformin, DPP-IV-inhibitor, sulfonylureas, GLP-1 mimetics) or/and insulin
- For at least 4 weeks prior entry to the study and during the study:
    - stable metabolic control (constant medication regarding glucose, lipid and uric acid metabolism)
    - stable body weight
    - constant dietary habits and physical activity levels
- HbA1c concentration <9.5%
- Serum total cholesterol (TC) <300 mg/dl (<7.76 mmol/l)
- Serum triglycerides (TG) <500 mg/dl (<5.7 mmol/l)
- Serum creatinine <2.5 mg/dl (<221 µmol/l)

Exclusion criteria

- Men and women with Type 1 diabetes
- Smoking
- Intention to change dietary habits, frequency of physical activity or body weight during or 4 weeks before entry to the study
- Glitazone intake
- Intake of fish oil capsules and other fatty acids
- Pregnant and breastfeeding women
- Participation in another clinical trial
- Gastrointestinal malabsorption (pancreatic insufficiency, steatorrhea, short bowel syndrome)
- Cardiovascular disease, defined by New York Heart Association Functional Classification ≥III
- Liver disease (transaminase-threshold ≥2.5 times increased)
- Chronic renal failure (dialysis patients or creatinine >2.5 mg/dl)
- Organ transplantation, Cancer, HIV
- Intake of systemic steroids
- Drug and alcohol abuse (≥80 g/d)
- Methadone intake during the last 2 years

Non-diabetic individuals had to be to be free of diabetes (based on definitions of ADA (ADA, 2009)) and were not allowed to take any glucose lowering drugs. Otherwise the same exclusion criteria were applied.

The inclusion criteria were fulfilled by 151 subjects. 120 gave their written consent. Out of these, 21 withdrew because of health problems unrelated to the study, digestive discomfort or scheduling conflicts. Two participants of the non-diabetic group were excluded because of elevated fasting glucose levels.

4.2 Anthropometry and questionnaires

Waist circumference, body height (stadiometer: Seca, Modell 214, Hamburg, Germany) and body weight (digital scale: Seca, Bella 840, Hamburg, Germany) were measured. Body mass index (BMI) was calculated as kg/m^2.

At each study visit, three blood pressure measurements (BpTRU Medical Devices, Coquitlam, BC, Canada) with 2 min intervals in between (the mean of the last two measurements was used) were obtained after at least 5 min rest with the subject in a seated position.

The Framingham general cardiovascular risk (D'Agostino Sr et al., 2008) was estimated, using following variables: age, sex, tobacco use, treated/untreated systolic blood pressure, diabetes and TC, HDL-cholesterol.

Participants had to complete and a food frequency questionnaire (FFQ) at baseline and week 16. A dietary diary and a blood glucose profile (only diabetic individuals) had to be filled in every day.

4.3 Blood sampling and isolation of peripheral blood mononuclear cells

Venous blood samples were obtained after an overnight fast using heparin, ethylene-ediaminetetraacetic acid (EDTA) or serum tubes (Becton Dickinson, Schwechat, Austria). After centrifugation, serum and plasma were aliquoted, used fresh or frozen at -80°C for further analysis. Erythrocytes were washed three times with isotonic phosphate buffer, aliquoted and stored at -80°C.

Peripheral blood mononuclear cells (PBMCs) were isolated using Cell Preparation Tubes (Becton Dickinson, Schwechat, Austria). They were separated by centrifugation according to the manufacturer's instructions and washed twice with cold phosphate buffered saline (PBS).

4.4 Detection of oxidative damage to DNA in peripheral blood mononuclear cells (Comet assay)

Oxidative damage to DNA, FPG-sensitive sites and resistance against H_2O_2 induced DNA damage were measured with the single-cell gel electrophoresis assay (comet assay) (Azqueta et al., 2009). Briefly, a freshly prepared suspension of PBMCs (approximately 1 x 10^6 cells/ml in PBS) was mixed with 1% low melting point agarose and put on agarose coated slides (1% normal melting agarose). Four slides were prepared from each cell suspension: "lysis", "buffer", "FPG" and "H_2O_2". All slides were put in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris base plus 1% Triton X-100 added before use, pH 10) for at least one hour. Only "H_2O_2" slides were put in 100 µM H_2O_2 solution for 5 min at 4°C before lysis. After lysis, the "buffer" and "FPG" slides were washed three times with enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8) and incubated with 50 µl of enzyme buffer ("buffer" slide) or FPG solution ("FPG" slide) for 30 min at 37°C in a moist box. Slides were put in an electrophoresis tank (CSL-COM40, Biozym, Austria) containing electrophoresis solution (0.3 M NaOH, 1 mM EDTA). After 20 min of unwinding and 30 min of electrophoresis (25 V, 300 mA at 4°C, pH >13) slides were washed with PBS and dried at room temperature.

Slides were stained with ethidium bromide (20 µg/ml) and the degree of DNA damage was quantified using a fluorescence microscope coupled with an imaging analysis system (Komet 5.5, Kinetinc Imaging, Liverpool, UK). For each sample, two replicate gels were analysed and the mean % DNA in tail of 50 comets per gel was calculated. The net amount of FPG-sensitive sites was determined by calculating the difference between the obtained DNA damage after FPG and buffer treatment.

4.5 Detection of oxidative damage to DNA and RNA in urine

Urine samples were collected the day of blood sampling, aliquoted and stored at -20°C until analysis. 8-oxodG and 8-oxoGuo were measured at the Laboratory of Clinical Pharmacology, Rigshospitalet, in Copenhagen, using a validated method for ultraperformance liquid chromatography (UPLC) and tandem mass spectrometry (Henriksen et al., 2009). In brief, the chromatographic separation was performed on an Acquity UPLC system (Waters, Milford, MA, USA), using an Acquity UPLC BEH Shield RP18 column (1.7µm, 2.1x100mm) and a gradient of A: 2.5 mM ammonium acetate (pH 5) and B: Acetonitrile. The MS/MS detection was performed on an API 3000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) using electrospray

ionisation operated in the positive mode. 15N5-8oxoGuo and 15N5-8oxodG were applied as internal standards. Two specific product-ions were measured from each analyte to ensure correct identification and quantification. 8-oxodG and 8-oxoGuo were normalized against urinary creatinine concentration determined by the Jaffe reaction.

4.6 Micronucleus assay in lymphocytes

The CBMN assay was performed according to the protocol of Fenech (Fenech, 2007). A concentration of 1×10^6 cells/ml in culture medium was stimulated to mitotic division with phytohemagglutinin (PHA; PAA, Austria) and incubated at 37°C, 5% CO_2. 44 hrs after PHA stimulation, cytocalasin-B (Sigma Aldrich, Austria) was added to block cytokinesis. After a total of 72 hrs of incubation, cells were transferred to slides using cytocentrifuge (Shandon Cytospin 3) and stained (Diff-Qick; Medion Diagnostics, Switzerland). For each subject duplicates were performed and two slides of each duplicate were prepared. The frequency of binucleated cells (BN) with micronucleus (MN), nuclear buds (NBuds), nucleoplasmic bridges (NPBs) was scored in 2000 BN according to published criteria (Fenech, 2007, Fenech et al., 2003). Furthermore, the number of necrotic and apoptotic cells as well as the nuclear division index (NDI) (Fenech, 2007) were determined.

4.7 Micronucleus assay in buccal cells

Cells were collected with toothbrushes from both cheeks after subjects rinsed their mouths with tap water. Subsequently, slides were prepared, stained and scored according to the method of Thomas et al. (Thomas et al., 2009). Cell suspensions were diluted to a concentration of >80,000 cells/ml. 120 µl of cell suspension were transferred to slides by cytocentrifugation (Shandon Cytocentrifuge Cytospine 4) and fixed with cold methanol (80%) for 30 min and ≥2,000 cells/sample were evaluated.

For Feulgen staining, cells were placed in beakers with 5.0 M HCl at room temperature for 15 min, rinsed with distilled water (15 min) and subsequently stained with Schiff's reagent (90 min). Cells were scored under bright field with 400-fold magnification using oil immersion with Eclipse E 600 microscope (Nikon, Tokyo, Japan) and then confirmed as positive under fluorescence. MNi were scored in a combination of both basal and differentiated cells according to the criteria defined by Thomas et al. (Thomas et al., 2009).

Differentiated buccal cells were scored for biomarkers of genomic damage (BN with MN, total number of MN, BN and broken eggs (BE)) and biomarkers for cell death (kariolysis (KL), karyorrhexis and condensed chromatin cells (KR+CC), and pycnotic cells (P)). Since KR and CC cells are difficult to discriminate, they were scored together.

4.8 Vitamins, antioxidants, fatty acids and homocysteine

The fatty acid profile in plasma was determined by a gas chromatograph equipped with a flame ionisation detector (Wagner et al., 2000). Identification of fatty acids was based on the samples' retention times compared to a 37 Component FAME Mix standard (Supelco, Bellefonte, USA). TotalChrom Workstation 6.3.0, Perkin Elmer was used for peak integration.

Plasma concentrations of vitamins K, α- and γ-tocopherol, retinol, lutein, α- and β-carotene were determined by reverse-phase high-performance liquid chromatography (HPLC) (Jakob and Elmadfa, 1995). Each vitamin was quantitated on the basis of peak area using a calibration curve generated from standard solutions.

Plasma concentrations of vitamin B12 and folic acid were measured according to routine diagnostic tests on a Siemens Immulite 2000 analyser (Siemens Healthcare Diagnostics, Tarrytown, USA) at the laboratory of the Health Centre South, Vienna using a chemiluminescent enzyme immunoassay. Folic acid in erythrocytes was measured with radioimmunoassay. Standard curves were drawn and concentrations calculated according to the protocol published by the kit producer (MP Biomedicals, Germany).

Plasma homocysteine was measured by reversed-phase HPLC with fluorescence detector (emission wavelength: 515 nm; excitation wavelength: 385 nm) on a LiChrosphere column (5 µm, 125 x 4 mm; Merck, Hitachi, LaChrom, Austria). Potassium hydrogenphosphate buffer with 4% acetonitrile was used as mobile phase (Majchrzak et al., 2007).

4.9 Biochemical analyses

Glycosylated haemoglobin, fasting plasma glucose, insulin, TC, high density lipoprotein (HDL), low density lipoprotein (LDL), and TG were measured at the laboratory of the Health Centre South, Vienna.

Glycosylated haemoglobin was analysed in whole blood by HPLC (Automated Glycohaemoglobin Analyser HLC-723G8; Tosoh, Tokyo, Japan). Fasting plasma glucose was determined enzymatically by the hexokinase method (Aeroset, Abbott Diagnostics, Illinois, USA) and plasma insulin

concentrations were measured on an Immulite 2000 immunochemistry system using reagents and calibrator obtained from the instrument supplier (Siemens Medical Solutions Diagnostics, Flanders, USA). The principle of the method is a solid-phase, two-site chemiluminescent immunometric assay. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as the product of fasting plasma glucose (mmol/L) and insulin (μU/ml) concentrations, divided by 22.5 (Matthews et al., 1985).

Serum TC, HDL-cholesterol, and TG levels were measured enzymatically by an automated method (Aeroset, Abbott Laboratories, North Chicago, IL, USA) using commercial kits (Abbott). LDL-cholesterol concentrations were calculated according to the Friedewald formula.

Adiponectin was measured with a magnetic bead-based assay (Bio-Plex Pro diabetes assay, Bio-Rad Laboratories, Inc.) and a Bio-Plex array reader using Bio-Plex ManagerTM Software 4.1.1.

4.10 Oil analyses

The fatty acid pattern and tocopherol concentrations of the plant oil were determined with the same method as described in section 4.8. Sterol content of the plant oil was analysed by capillary gas chromatography at the Department of Food and Environmental Sciences, Helsinki, Finland (Piironen et al., 2002). The concentration of the given sterols or stanols (Table 2) is the sum of their free and esterified forms.

4.11 Statistical analyses

Statistical analyses were performed using SPSS (versions 17.0 – 20) for Windows (SPSS, Chicago, IL, USA). Normal distribution within the data set was tested by histograms and the Kolmogorov-Smirnov test. Independent samples T-test (for parametric data) or Mann-Whitney U-test (for non-parametric data) were conducted to assess differences between two groups. Multiple group comparisons were performed with one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test or Kruskal Wallis H-test if the assumptions of normality or homogeneity of variance were not met.

The effect of the intervention was assessed by (i) comparing baseline values with values after 4, 8 or 16 weeks of intervention and (ii) by comparing the change after 4, 8 or 16 weeks of intervention between the two treatment groups. Pearson correlation and Spearman rank correlation were used to evaluate the association between variables. Results were considered significant at $P < 0.05$.

5 Summary of Results and Discussion

5.1 Comparisons of baseline data between diabetic and non-diabetic individuals (Paper I – III)

Diabetic individuals were suffering from their disease on average for 11.3±10.5 years. They had significantly higher levels of fasting plasma glucose, HbA1c, waist circumference and BMI compared to their non-diabetic counterparts (Table 3). Regarding levels of **oxidative damage to DNA (Paper I)** measured by comet assay (lysis), no significant differences between diabetic and non-diabetic individuals were observed. Levels of FPG-sensitive sites were significantly higher in diabetic participants and individuals in the highest tertile of fasting plasma glucose (>8.6 mmol/l) had significantly higher level of FPG-sensitive sites compared to individuals in the lowest tertile (<6.39 mmol/l) (FPG-sensitive sites in the highest and lowest tertile: 2.85±1.99 vs. 4.47±2.95% DNA in tail, respectively).

Global DNA oxidation in urine (8-oxodG) did not differ between diabetic and non-diabetic individuals. In contrast, levels of 8-oxoGuo, a biomarker for global RNA oxidation which was recently suggested as a clinical biomarker for the assessment of mortality risk in newly diagnosed T2DM patients (Broedbaek et al., 2011a), were significantly higher in diabetic individuals compared to non-diabetic participants (Table 3). Furthermore, subjects with HbA1c concentrations ≥6.5% had significantly higher global RNA oxidation compared to subjects with HbA1c <6.5% (8-oxoGuo: 3.69±1.51 vs. 3.04±0.85 nmol/mmol creatinine, respectively).

Rates of **genomic damage in lymphocytes (Paper II)** did not differ between diabetic and non-diabetic individuals. The significant correlation between MN frequency and fasting plasma glucose levels ($r = 0.283$, $P = 0.006$, adjusted for sex) suggests an increase in chromosomal damage, manifested as increased MN frequency, with worsening of glycemic control. The importance of proper blood glucose control could also be confirmed by the significantly lower levels of fasting plasma glucose and HbA1c levels in diabetic subjects in the low 50^{th} percentile of MN frequency relative to those in the high 50^{th} percentile. Furthermore, diabetic subjects with high MN frequency showed significantly higher risk for CVDs compared to diabetic participants with low MN frequency. Non-diabetic participants with high MN frequency had significantly lower plasma vitamin B12 levels compared to non-diabetic individuals with low MN frequency. No associations with plasma and erythrocyte folate were identified.

In contrast to the results obtained from lymphocytes, **MN frequency in buccal cells (Paper III)** was significantly higher in diabetic compared to non-diabetic individuals (Table 3). Furthermore, associations between waist circumference, glucose metabolism, CVD-risk and buccal MN frequency were observed. Participants in the highest tertile of waist circumference, fasting plasma glucose, HbA1c and CVD-risk had significantly higher MN frequency (+40%, +55%, +41%, +39%, respectively) compared to participants in the lowest tertile. A trend (P <0.1) for significantly different MN levels between participants in the highest and lowest tertile of insulin and HOMA-IR was observed. No associations between vitamin B12, plasma folate, red blood cell folate, homocysteine and MN frequency were observed.

Table 3. Baseline characteristics of non-diabetic and diabetic individuals

	Non-diabetic	Diabetic
Number (male/female)	21 (6/15)	76 (34/42)
Age (years)	62.7 ± 6.30	65.1 ± 7.47
BMI (kg/m^2)	28.4 ± 3.84	33.6 ± 6.26*
Waist circumference (cm)	96.8 ± 10.0	113 ± 15.0*
Fasting plasma glucose (mmol/l)	5.63 ± 0.50	8.52 ± 2.20*
HbA1c (%)	5.79 ± 0.26	7.55 ± 0.89*
Insulin (pmol/l)	48.0 ± 38.6	137 ± 153*
HOMA-IR	1.97 ± 1.34	7.55 ± 9.42*
Lyse (% DNA in tail)	5.19 ± 1.66	5.45 ± 2.16
FPG-sensitive sites (% DNA in tail)	2.81 ± 1.47	3.92 ± 2.67*
8-oxoGuo (nmol/mmol creatinine)	2.93 ± 0.62	3.67 ± 1.49*
8-oxodG (nmol/mmol creatinine)	2.00 ± 0.75	2.11 ± 0.81*
MNi in lymphocytes (‰)	23.0 ± 9.08	21.4 ± 7.79
MNi in buccal cells (‰)	0.28 ± 0.29	0.58 ± 0.30*

*Significantly different from non-diabetic individuals

In the present study, oxidative DNA damage (Comet Assay) and MN frequency in lymphocytes did not differ between diabetic and non-diabetic individuals. Studies, which have investigated this issue have reported conflicting results (Arif et al., 2010, Ibarra-Costilla et al., 2010, Lodovici et al., 2008, Pitozzi et al., 2003, Song et al., 2007, Martínez-Pérez et al., 2007) which might reflect the heterogeneity of the disease diabetes itself.

On the contrary, levels of FPG-sensitive sites, 8-oxoGuo and MN frequency in buccal cells differed significantly between diabetic and non-diabetic participants. Furthermore, CVD-risk, which is increased in T2DM patients (Hu et al., 2005, Stamler et al., 1993), was associated with MN frequency in lymphocytes and buccal cells. Scientific evidence suggests that genomic instability is a relevant contributor to atherosclerosis (Mercer et al., 2010) and patients with coronary artery disease have higher MN frequency in lymphocytes (Botto et al., 2001). Mercer et al. suggested that persistent DNA damage in plaque cells changes the ratio of cell proliferation and apoptosis and promotes the risk of atherosclerosis (Mercer et al., 2010).

Also glucose metabolism was significantly associated with biomarkers of oxidative damage to purines and RNA (FPG-sensitive sites and 8-oxoGuo, respectively) and biomarkers of chromosomal damage (MN frequency in lymphocytes and buccal cells). This underpins the importance of good glycemic control in diabetic patients to avoid secondary complications.

5.2 Compliance during the intervention trial (Paper I + Paper II)

To asses dietary compliance the fatty acid profile and levels of γ-tocopherol (for the oil intake) and carotenoids, vitamin K and folate (for the vegetable intake) were measured. In subjects of the intervention group plasma levels of linoleic (+13%) and linolenic acid (+60%), γ-tocopherol (+37%), lutein (+58%), α-carotene (+92%), β-carotene (+53%), vitamin K (+154%) (all in **Paper I**), plasma folate (+13%) and red blood cell folate (+10%) (**Paper II**) were significantly increased after 8 weeks of intervention with vegetables and oil compared to baseline. None of the compounds changed significantly in the information group, apart from β-carotene showing a significant increase after 8 weeks (+31%; Figure 7). The changes in all the compliance markers were significantly higher in the intervention group compared with the information group, confirming good dietary compliance.

In figure 6 and figure 7 changes in selected compliance markers for oil and vegetable intake are presented, showing the changes in diabetic and non-diabetic individuals of the information and intervention group.

Since subjects were instructed to consume their habitual diet after the intervention period, most of the compliance markers returned to their baseline levels at week 16. Only linolenic acid and α-carotene remained significantly higher in participants of the intervention group compared to baseline. However, there were no significant differences in compliance markers between intervention and information group at week 16.

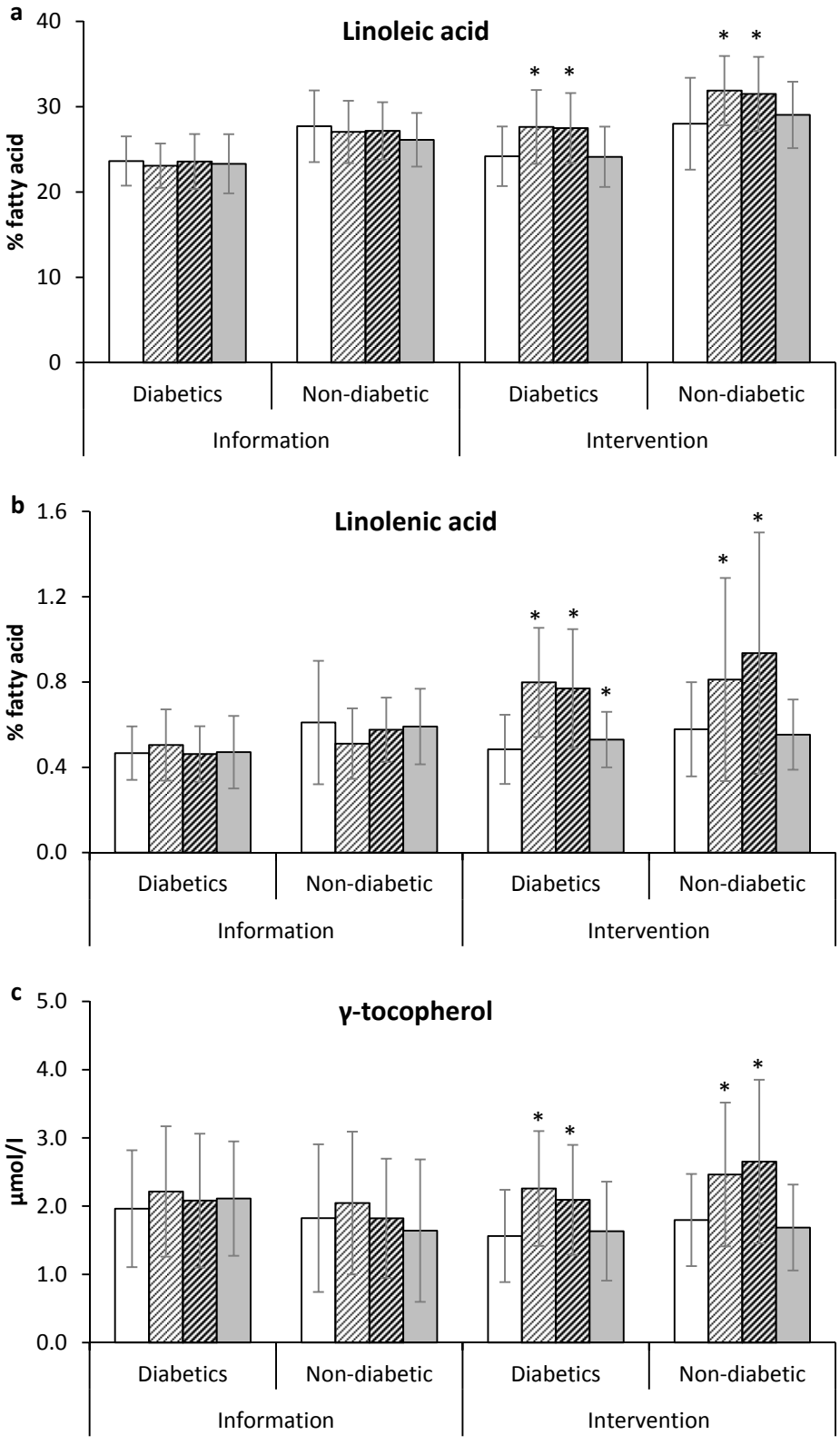


Figure 6. Changes in compliance markers of PUFA-rich walnut oil intake (a: linoleic acid, b: linolenic acid, c: γ-tocopherol). *Significantly different from baseline of the corresponding group. □ baseline ▨ after 4 weeks ▨ after 8 weeks (end of intervention) ▨ after 16 weeks

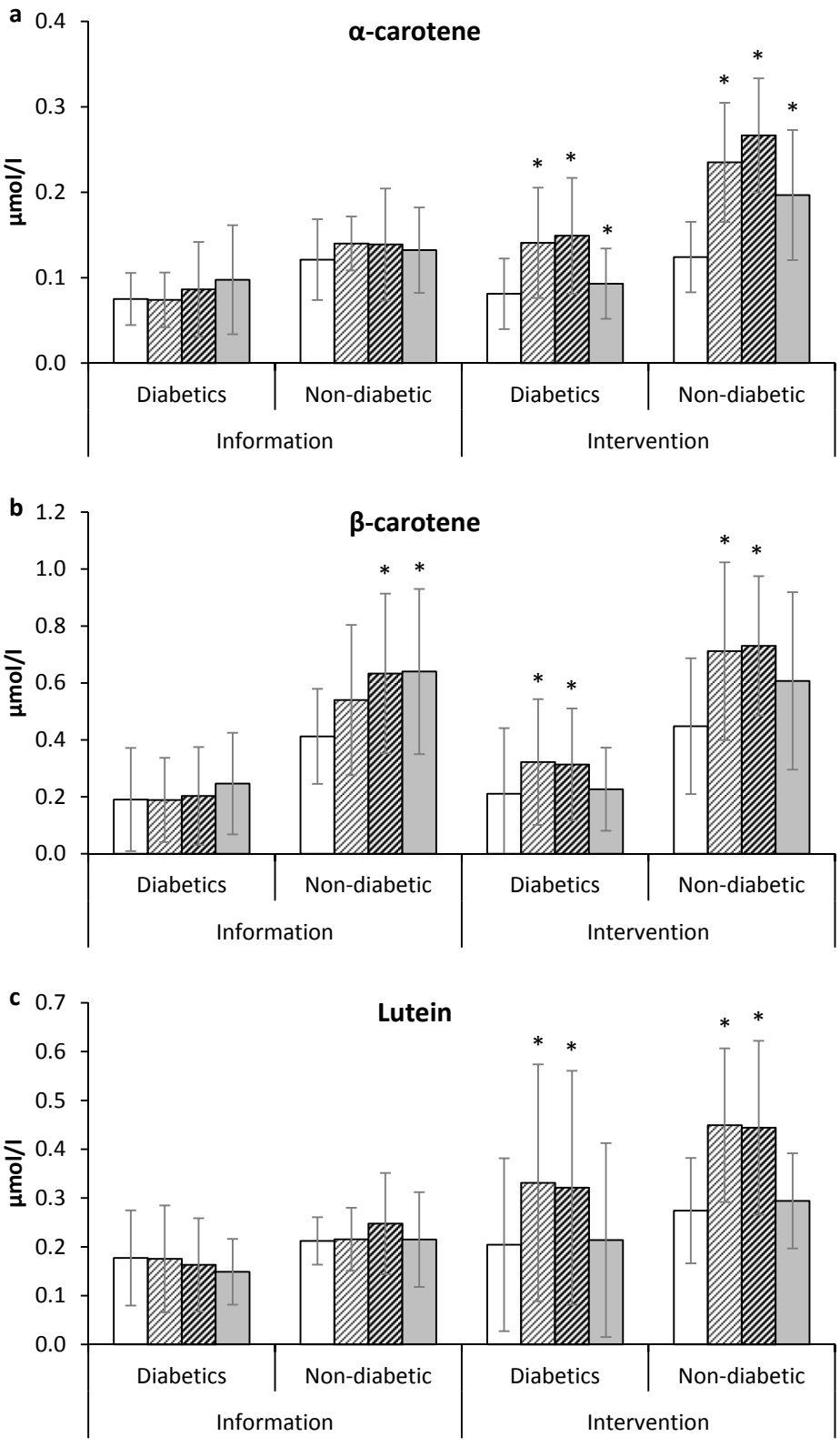


Figure 7. Changes in selected vegetable compliance parameters (a: α-carotene, b: β-carotene, c: lutein. *Significantly different from baseline value of the corresponding group.

□ baseline ▨ after 4 weeks ▨ after 8 weeks (end of intervention) ▨ after 16 weeks

5.3 Impact of the intervention on glycemic control (Paper I)

Significant reductions in HbA1c levels were observed after 4 and 8 weeks of intervention with oil and vegetables in diabetic subjects (Table 4), while HbA1c levels remained constant in non-diabetic individuals. In the information group, HbA1c levels were not significantly changed after 4 weeks, but significantly improved in diabetic individuals after 8 weeks. Consequently, changes in HbA1c levels were not significantly different between the two treatment groups.

After return to the usual diet (week 16), diabetic individuals of the intervention group still tended to have lower HbA1c levels than at baseline (P <0.1). However, in none of the groups HbA1c was significantly different from baseline.

Fasting plasma glucose was significantly reduced in diabetic individuals after 4 weeks of intervention with vegetables and plant oil, but not after 8 weeks. In all other groups fasting plasma glucose levels were not significantly altered.

Table 4. Changes in HbA1c and fasting plasma glucose in diabetic and non-diabetic participants of the information and intervention group

	baseline	week 4	week 8	week 16
HbA1c (%)				
Intervention				
Diabetic	7.58 ± 0.93	7.38 ± 0.82*	7.36 ± 0.80*	7.40 ± 0.97
Non-Diabetic	5.83 ± 0.24	5.79 ± 0.33	5.79 ± 0.47	5.83 ± 0.34
Information				
Diabetic	7.48 ± 0.80	7.38 ± 0.88	7.25 ± 0.89*	7.44 ± 0.87
Non-Diabetic	5.71 ± 0.28	5.74 ± 0.27	5.73 ± 0.30	5.76 ± 0.26
Fasting glucose (mmol/l)				
Intervention				
Diabetic	8.68 ± 2.28	8.51 ± 1.96*	8.58 ± 2.08	8.16 ± 2.10
Non-Diabetic	5.67 ± 0.47	5.51 ± 0.27	5.65 ± 0.48	5.50 ± 0.45
Information				
Diabetic	8.15 ± 2.00	7.95 ± 1.77	8.32 ± 2.11	8.70 ± 2.12
Non-Diabetic	5.58 ± 0.55	5.55 ± 0.57	5.52 ± 0.61	5.60 ± 0.47

*Significantly different from baseline of the corresponding group.

The present reduction in HbA1c, which is the long term marker of glucose control and reflects the average glucose levels during the previous 8 to 12 weeks, underlines the importance of diet in the treatment of T2DM. Interestingly, glycemic control can be improved as much by information, possibly leading to nutritional changes, as by a dietary intervention.

5.4 Effect of vegetables and PUFA-rich plant oil on levels of oxidative damage to DNA (Paper I)

Daily vegetable and oil consumption reduced DNA strand breaks in diabetic individuals after 4 and 8 weeks of intervention compared to baseline. No effects on levels of DNA strand breaks were observed in healthy participants of the intervention group or in subjects of the information group (Figure 8).

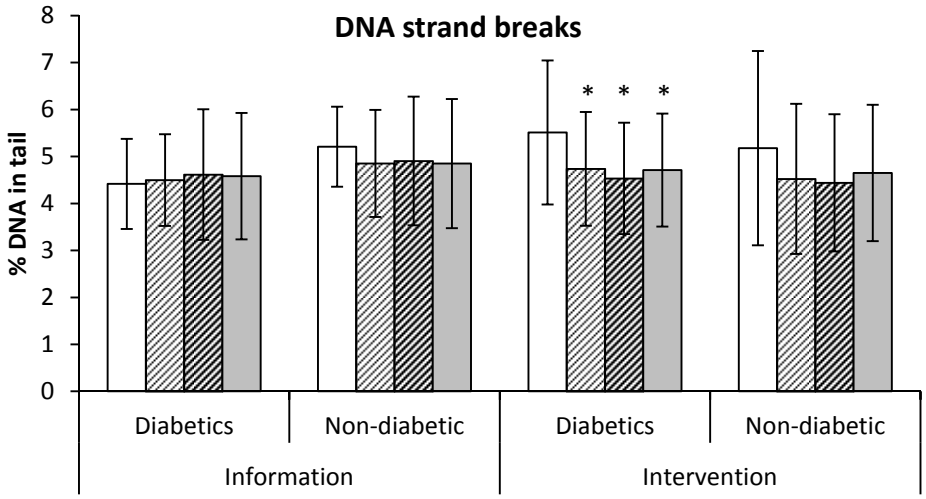


Figure 8. Changes in oxidative damage to DNA assessed by comet assay in participants of the information and intervention group. *Significantly different from baseline of the corresponding group. □ baseline ▨ after 4 weeks ▨ after 8 weeks (end of intervention) ▨ after 16 weeks

FPG-sensitive sites were significantly reduced in diabetic individuals of the intervention group after 4 weeks, whereas after 8 weeks, levels were not significantly different from baseline (baseline: 3.87 ± 2.43, week 4: 3.13 ± 2.32, week 8: 3.39 ± 2.33% DNA in tail).

The reductions in DNA strand breaks, FPG-sensitive sites after 4 and 8 weeks were not significantly different between information and intervention group. Levels of H_2O_2-sensitive sites, 8-oxodG and 8-oxoGuo, markers for DNA and RNA oxidation, respectively, were not affected by

the dietary intervention. There was no difference in 8-oxodG and 8-oxoGuo excretion or in H_2O_2-sensitivity after 4 and 8 weeks between the two treatment groups.

After return to the usual diet, levels of FPG- and H_2O_2-sensitive sites were not significantly different from baseline in none of the groups. Levels of strand breaks increased at week 16, but were still significantly lower compared to baseline in diabetic individuals of the intervention group (Figure 8).

To the best of our knowledge, no data about the impact of vegetables and plant oil on DNA damage in T2DM subjects are published. Supplementation trials with vitamin E (Şardaş et al., 2001) or flavonols (Lean et al., 1999) are consistent with our findings and showed a reduction in strand breaks in T2DM subjects. Intervention studies in healthy individuals with a vegetable/fruit concentrate (Van Den Berg et al., 2001), 600 g of fruit and vegetables (Møller et al., 2003), 200 g of cooked minced carrots (Astley et al., 2004), a tomato-based drink (Riso et al., 2006), 2, 5, or 8 servings/day of vegetables and fruit (Briviba et al., 2008), 300 g of Brussels sprouts (Hoelzl et al., 2008) or 225 g of spinach (Moser et al., 2011) reported no effect on DNA strand breaks.

The discrepancy in effects of the intervention between diabetic and non-diabetic individuals in the present study might be due to significantly lower baseline levels of α- and β-carotene in diabetic subjects compared to non-diabetic individuals, indicating that subjects with low antioxidant status benefit more from the intervention. We also observed a significantly weaker increase in plasma α- and β-carotene after 8 weeks of intervention in diabetic individuals compared to non-diabetic subjects, which might reflect the use of the mentioned antioxidants as radical scavengers.

These data suggest that a healthy diet, rich in vegetables and a considerable amount of PUFA, replacing SFA, decreases levels of DNA strand breaks. This positive effect might be related to the reduction in HbA1c and the improvement in antioxidant status, strengthening the idea that diet quality is important in treatment of T2DM.

5.5 Effect of vegetables and PUFA-rich plant oil on levels of chromosomal damage in lymphocytes (Paper II) and buccal cells (Paper III)

The intervention with vegetables and plant oil did not affect MN frequency in lymphocytes and buccal cells. In lymphocytes, only apoptosis was slightly but significantly increased (Table 5) in participants of the intervention group. However, considering the percentage of apoptotic cells, this increase might not be of biological significance. The results obtained within this trial were in the normal range of healthy subjects (Fenech, 2007).

Table 5. Changes in chromosomal stability of lymphocytes after 8 weeks in the intervention and information group

	Information			Intervention		
	baseline	week 8	P^1	baseline	week 8	P^1
BN with MN/1000 BN			0.871[2]			0.516[2]
Diabetic	23.0±8.51	23.7±10.8		20.7±7.44	22.5±9.35	
Non-Diabetic	22.5±4.68	21.8±5.05		23.3±11.3	24.2±14.9	
BN with NPB/1000 BN			0.204			0.236
Diabetic	1.9±1.48	2.12±1.64		1.56±1.24	1.85±1.39	
Non-Diabetic	1.44±0.62	2.06±0.95		2.01±1.36	2.42±1.41	
BN with NBuds/1000 BN			0.900			0.152
Diabetic	5.08±2.97	4.73±2.33		3.92±2.03	5.01±2.66	
Non-Diabetic	2.69±1.81	3.22±1.95		6.38±5.02	5.47±3.85	
Apoptosis (%)			0.939			0.006
Diabetic	1.44±0.69	1.28±0.47		1.2±0.5	1.47±0.56	
Non-Diabetic	1.14±0.37	1.42±0.6		1.46±0.49	1.46±0.49	
Necrosis (%)			0.270			0.569
Diabetic	1.28±0.75	1.29±0.46		1.41±0.76	1.47±0.67	
Non-Diabetic	0.95±0.51	1.03±0.42		1.56±0.81	1.23±0.41	

[1] *P*-values refer to difference between baseline and week 8 in the information and intervention group (diabetic and non-diabetic individuals together) [2] adjusted for sex.

In buccal cells, the number of BNC and KR + CC was significantly reduced (Table 6). It is unlikely that this reduction is due to the intervention since both markers were reduced in the information group as well. PC were only reduced in participants of the intervention group and changes after 8 weeks were significantly different between the two treatment groups.

Table 6. Changes in chromosomal stability of buccal cells after 8 weeks in the intervention and information group

	Information			Intervention		
	baseline	week 8	P^1	baseline	week 8	P^1
MN cells (‰)			0.816			0.331
Diabetic	0.53±0.29	0.52±0.36		0.61±0.30	0.51±0.29	
Non-Diabetic	0.22±0.26	0.27±0.26		0.32±0.31	0.40±0.36	
BNC (‰)			0.001			0.000
Diabetic	21.3±13.90	15.7±7.42		21.5±12.78	15.0±7.50	
Non-Diabetic	22.8±9.81	13.6±8.45		22.0±17.31	13.3±6.26	
BE (‰)			0.200			0.493
Diabetic	0.62±0.56	0.49±1.29		0.76±0.80	0.71±0.82	
Non-Diabetic	0.16±0.33	0.45±0.36		0.90±1.13	0.62±0.64	
KR +CC (‰)			0.038			0.005
Diabetic	24.3±19.4	15.4±11.1		23.0±15.9	18.6±15.9	
Non-Diabetic	26.7±19.2	23.8±22.0		24.5±22.5	21.3±15.5	
KL (‰)			0.597			0.159
Diabetic	63.3±77.9	60.0±76.9		56.0±65.7	49.3±54.7	
Non-Diabetic	54.3±76.1	41.5±52.7		57.4±76.7	43.9±74.8	
PC (‰)			0.554			0.001
Diabetic	0.74±0.94	0.84±0.88		1.24±1.43	0.59±0.59	
Non-Diabetic	0.49±0.51	0.42±0.50		0.80±0.47	0.57±0.40	

[1] *P*-values refer to difference between baseline and week 8 in the information and intervention group (diabetic and non-diabetic individuals together)

Based on an intervention study with folate and vitamin B12 Fenech et al. suggest red blood cell folate concentrations >700 nmol/l, plasma vitamin B12 >300 pmol/l and homocysteine levels <7.5 µmol/l to minimize micronucleus formation in lymphocytes (Fenech, 2012, Fenech et al., 1998). Even though folic acid was increased within this trial, only 6% of participants assigned to the intervention group had red blood cell folate levels >700 nmol/l. The required homocysteine and B12 levels were reached by 2% and 26% of subjects of the intervention group, respectively.

Intervention studies with multiple antioxidants supplementing 1000 mg ascorbic acid and 335.5 mg vitamin E (Schneider et al., 2001), a mixed supplement consisting of 100 mg α-tocopherol, 6 mg β-carotene, 100 mg vitamin C and 50 µg selenium (Smolková et al., 2004), or a vitamin-antioxidant mixture containing 3 mg vitamin A, 30 mg α–tocopherol, 150 mg ascorbic acid, 15 mg β-carotene, 0.2 mg folic acid and 75 mg rutin (Gaziev et al., 1996) reported positive effects on MN frequency in lymphocytes. Also buccal MN levels were significantly reduced after supplementation with 516 µg of folate per day (Titenko-Holland et al., 1998).

In this trial, no supplements were administered. Antioxidant and vitamin concentrations were increased by providing whole foods which, compared to supplements, naturally contain lower concentrations of micronutrients.

6 Summary and Main Findings

Diabetes is a complex metabolic disorder associated with increased risk of micro- and macrovascular complications (Brownlee, 2001) and site-specific cancers compared to healthy individuals (Vigneri et al., 2009). The association between diabetes and associated complications is multifactorial, as factors like obesity, quality of metabolic control, medical treatment and lifestyle have major impact on the onset of complications. Diet is a cornerstone in the treatment of T2DM and contributes essentially to the achievement of optimal blood glucose control.

In the present study oxidative damage to DNA and chromosomal damage in lymphocytes and buccal cells were compared between diabetic and healthy individuals. Furthermore, the impact of a dietary intervention with vegetables and PUFA-rich plant oil on the above mentioned biomarkers was assessed and results can be summarized as follows:

- Levels of DNA strand breaks were not significantly different between diabetic and non-diabetic individuals. However, levels of FPG-sensitive sites were significantly higher in T2DM patients and were positively associated with fasting plasma glucose, suggesting that FPG-sensitive sites specifically reflect damage resulting from hyperglycaemia.
- 8-oxoGuo suggested as biomarker to assess mortality risk in newly diagnosed T2DM patients (Broedbaek et al., 2011a), was significantly higher in diabetic individuals compared with non-diabetic participants.
- Levels of chromosomal damage (MN frequency) in lymphocytes were not significantly different between diabetic and non-diabetic individuals and were within the normal range (Fenech, 2007). Associations between glucose metabolism, CVD-risk, vitamin B12 and MN frequency were observed.
- Levels of chromosomal damage (MN frequency) in buccal cells were significantly higher in diabetic individuals. Similar to lymphocytes, associations between glycemic control, CVD-risk, waist circumference and buccal MN frequency were observed.
- The dietary intervention with 300 g of vegetables led to following changes:
    - Increases in concentrations of vitamin E, vitamin K, α-, β-carotene, lutein, plasma and red blood cell folate, and essential fatty acids (linoleic- and linolenic acid).
    - Improvement in glycemic control, reflected in reduction of HbA1c.
    - Reduction DNA strand breaks in diabetic individuals.
    - No changes in levels of MN frequency in lymphocytes and buccal cells.

- Giving information to participants about the importance of a healthy diet led to following changes:
    - Significant increase in β-carotene.
    - Improvement in glycemic control, reflected in decreased levels of HbA1c.
    - No impact on oxidative damage to DNA or chromosomal damage.

7 References

ADA 2008. Nutrition recommendations and interventions for diabetes: A position statement of the American Diabetes Association. *Diabetes Care,* 31**,** S61-S78.

ADA 2009. Diagnosis and classification of diabetes mellitus. *Diabetes Care,* 32**,** S62-S67.

ADA 2012. Diagnosis and classification of diabetes mellitus. *Diabetes Care,* 35**,** S64-S71.

ALIMOVA, I. N., LIU, B., FAN, Z., EDGERTON, S. M., DILLON, T., LIND, S. E. & THOR, A. D. 2009. Metformin inhibits breast cancer cell growth, colony formation and induces cell cycle arrest in vitro. *Cell Cycle,* 8**,** 909-915.

AMES, B. N., GOLD, L. S. & WILLETT, W. C. 1995. The causes and prevention of cancer. *Proceedings of the National Academy of Sciences of the United States of America,* 92**,** 5258-5265.

ANTUNA-PUENTE, B., FEVE, B., FELLAHI, S. & BASTARD, J. P. 2008. Adipokines: The missing link between insulin resistance and obesity. *Diabetes and Metabolism,* 34**,** 2-11.

ARCIDIACONO, B., IIRITANO, S., NOCERA, A., POSSIDENTE, K., NEVOLO, M. T., VENTURA, V., FOTI, D., CHIEFARI, E. & BRUNETTI, A. 2012. Insulin resistance and cancer risk: An overview of the pathogenetic mechanisms. *Experimental Diabetes Research,* 2012.

ARIF, M., ISLAM, M. R., WAISE, T. M. Z., HASSAN, F., MONDAL, S. I. & KABIR, Y. 2010. DNA damage and plasma antioxidant indices in Bangladeshi type 2 diabetic patients. *Diabetes and Metabolism,* 36**,** 51-57.

ARONOFF, S. L., BERKOWITZ, K., SHREINER, B. & WANT, L. 2004. Glucose metabolism and Regulation: Beyond Insulin and Glucagon. *Diabetes Spectrum,* 17**,** 183-190.

ARONSON, D. 2003. Cross-linking of glycated collagen in the pathogenesis of arterial and myocardial stiffening of aging and diabetes. *Journal of Hypertension,* 21**,** 3-12.

ARTWOHL, M., RODEN, M., HÖLZENBEIN, T., FREUDENTHALER, A., WALDHÄUSL, W. & BAUMGARTNER-PARZER, S. M. 2002. Modulation by leptin of proliferation and apoptosis in vascular endothelial cells. *International Journal of Obesity,* 26**,** 577-580.

ASTLEY, S. B., ELLIOTT, R. M., ARCHER, D. B. & SOUTHON, S. 2004. Evidence that dietary supplementation with carotenoids and carotenoid-rich foods modulates the DNA damage: Repair balance in human lymphocytes. *British Journal of Nutrition,* 91**,** 63-72.

AZQUETA, A., SHAPOSHNIKOV, S. & COLLINS, A. R. 2009. DNA oxidation: Investigating its key role in environmental mutagenesis with the comet assay. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis,* 674**,** 101-108.

BANSAL, D., BHANSALI, A., KAPIL, G., UNDELA, K. & TIWARI, P. 2012. Type 2 diabetes and risk of prostate cancer: a meta-analysis of observational studies. *Prostate Cancer and Prostatic Diseases*.

BAXTER, R. C. & TURTLE, J. R. 1978. Regulation of hepatic growth hormone receptors by insulin. *Biochemical and Biophysical Research Communications,* 84**,** 350-357.

BELFIORE, A. & MALAGUARNERA, R. 2011. Insulin receptor and cancer. *Endocrine-Related Cancer,* 18**,** R125-R147.

BELLAMY, L., CASAS, J.-P., HINGORANI, A. D. & WILLIAMS, D. 2009. Type 2 diabetes mellitus after gestational diabetes: a systematic review and meta-analysis. *The Lancet,* 373**,** 1773-1779.

BEN, Q., XU, M., NING, X., LIU, J., HONG, S., HUANG, W., ZHANG, H. & LI, Z. 2011. Diabetes mellitus and risk of pancreatic cancer: A meta-analysis of cohort studies. *European Journal of Cancer,* 47**,** 1928-1937.

BENECKE, H., FLIER, J. S. & MOLLER, D. E. 1992. Alternatively spliced variants of the insulin receptor protein. Expression in normal and diabetic human tissues. *Journal of Clinical Investigation,* 89**,** 2066-2070.

BODMER, M., MEIER, C., KRÄHENBÜHL, S., JICK, S. S. & MEIER, C. R. 2010. Long-term metformin use is associated with decreased risk of breast cancer. *Diabetes Care,* 33**,** 1304-1308.

BONASSI, S., ZNAOR, A., CEPPI, M., LANDO, C., CHANG, W. P., HOLLAND, N., KIRSCH-VOLDERS, M., ZEIGER, E., BAN, S., BARALE, R., BIGATTI, M. P., BOLOGNESI, C., CEBULSKA-WASILEWSKA, A., FABIANOVA, E., FUCIC, A., HAGMAR, L., JOKSIC, G., MARTELLI, A., MIGLIORE, L., MIRKOVA, E., SCARFI, M. R., ZIJNO, A., NORPPA, H. & FENECH, M. 2007. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis,* 28**,** 625-631.

BOTTO, N., RIZZA, A., COLOMBO, M. G., MAZZONE, A. M., MANFREDI, S., MASETTI, S., CLERICO, A., BIAGINI, A. & ANDREASSI, M. G. 2001. Evidence for DNA damage in patients with coronary artery disease. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis,* 493**,** 23-30.

BOWKER, S. L., MAJUMDAR, S. R., VEUGELERS, P. & JOHNSON, J. A. 2006. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. *Diabetes Care,* 29**,** 254-258.

BOYLE, P., BONIOL, M., KOECHLIN, A., ROBERTSON, C., VALENTINI, F., COPPENS, K., FAIRLEY, L. L., ZHENG, T., ZHANG, Y., PASTERK, M., SMANS, M., CURADO, M. P., MULLIE, P., GANDINI, S.,

BOTA, M., BOLLI, G. B., ROSENSTOCK, J. & AUTIER, P. 2012. Diabetes and breast cancer risk: A meta-analysis. *British Journal of Cancer,* 107**,** 1608-1617.

BRIVIBA, K., BUB, A., MÖSENEDER, J., SCHWERDTLE, T., HARTWIG, A., KULLING, S. & WATZL, B. 2008. No differences in DNA damage and antioxidant capacity between intervention groups of healthy, nonsmoking men receiving 2, 5, or 8 servings/day of vegetables and fruit. *Nutrition and Cancer,* 60**,** 164-170.

BROEDBAEK, K., SIERSMA, V., HENRIKSEN, T., WEIMANN, A., PETERSEN, M., ANDERSEN, J. T., JIMENEZ-SOLEM, E., HANSEN, L. J., HENRIKSEN, J. E., BONNEMA, S. J., DE FINE OLIVARIUS, N. & POULSEN, H. E. 2012. Association Between Urinary Markers of Nucleic Acid Oxidation and Mortality in Type 2 Diabetes: A population-based cohort study. *Diabetes Care,* 36, 669-76

BROEDBAEK, K., SIERSMA, V., HENRIKSEN, T., WEIMANN, A., PETERSEN, M., ANDERSEN, J. T., JIMENEZ-SOLEM, E., STOVGAARD, E. S., HANSEN, L. J., HENRIKSEN, J. E., BONNEMA, S. J., OLIVARIUS NDE, F. & POULSEN, H. E. 2011a. Urinary markers of nucleic acid oxidation and long-term mortality of newly diagnosed type 2 diabetic patients. *Diabetes Care,* 34**,** 2594-6.

BROEDBAEK, K., WEIMANN, A., STOVGAARD, E. S. & POULSEN, H. E. 2011b. Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine as a biomarker in type 2 diabetes. *Free Radical Biology and Medicine,* 51**,** 1473-1479.

BROWNLEE, M. 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature,* 414**,** 813-820.

BROWNLEE, M. 2005. The pathobiology of diabetic complications: A unifying mechanism. *Diabetes,* 54**,** 1615-1625.

BUTLER, A. E., JANSON, J., BONNER-WEIR, S., RITZEL, R., RIZZA, R. A. & BUTLER, P. C. 2003. β-cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. *Diabetes,* 52**,** 102-110.

CALLE, E. E. & KAAKS, R. 2004. Overweight, obesity and cancer: Epidemiological evidence and proposed mechanisms. *Nature Reviews Cancer,* 4**,** 579-591.

CANTRELL, L. A., ZHOU, C., MENDIVIL, A., MALLOY, K. M., GEHRIG, P. A. & BAE-JUMP, V. L. 2010. Metformin is a potent inhibitor of endometrial cancer cell proliferation-implications for a novel treatment strategy. *Gynecologic Oncology,* 116**,** 92-98.

CASTILLO, J. J., MULL, N., REAGAN, J. L., NEMR, S. & MITRI, J. 2012. Increased incidence of non-Hodgkin lymphoma, leukemia, and myeloma in patients with diabetes mellitus type 2: A meta-analysis of observational studies. *Blood,* 119**,** 4845-4850.

CERIELLO, A. & TESTA, R. 2009. Antioxidant anti-inflammatory treatment in type 2 diabetes. *Diabetes Care,* 32 Suppl 2**,** S232-236.

CHAN, B. T. Y. & LEE, A. V. 2008. Insulin receptor substrates (IRSs) and breast tumorigenesis. *Journal of Mammary Gland Biology and Neoplasia,* 13**,** 415-422.

CHOWDHURY, T. A. 2010. Diabetes and cancer. *QJM,* 103**,** 905-915.

COHEN, D. H. & LEROITH, D. 2012. Obesity, type 2 diabetes, and cancer: The insulin and IGF connection. *Endocrine-Related Cancer,* 19**,** F27-45.

COLHOUN, H. M. 2009. Use of insulin glargine and cancer incidence in Scotland: A study from the Scottish Diabetes Research Network Epidemiology Group. *Diabetologia,* 52**,** 1755-1765.

COLLINS, A. R., GEDIK, C. M., OLMEDILLA, B., SOUTHON, S. & BELLIZZI, M. 1998. Oxidative DNA damage measured in human lymphocytes: Large differences between sexes and between countries, and correlations with heart disease mortality rates. *FASEB Journal,* 12**,** 1397-1400.

COLLINS, A. R., OSCOZ, A. A., BRUNBORG, G., GAIVÃO, I., GIOVANNELLI, L., KRUSZEWSKI, M., SMITH, C. C. & ŠTĚTINA, R. 2008. The comet assay: topical issues. *Mutagenesis,* 23**,** 143-151.

COOKE, M. S., EVANS, M. D., DIZDAROGLU, M. & LUNEC, J. 2003. Oxidative DNA damage: mechanisms, mutation, and disease. *The FASEB Journal,* 17**,** 1195-1214.

COUGHLAN, M. T., THORBURN, D. R., PENFOLD, S. A., LASKOWSKI, A., HARCOURT, B. E., SOURRIS, K. C., TAN, A. L. Y., FUKAMI, K., THALLAS-BONKE, V., NAWROTH, P. P., BROWNLEE, M., BIERHAUS, A., COOPER, M. E. & FORBES, J. M. 2009. RAGE-Induced Cytosolic ROS Promote Mitochondrial Superoxide Generation in Diabetes. *Journal of the American Society of Nephrology,* 20**,** 742-752.

CURRIE, C. J., POOLE, C. D. & GALE, E. A. M. 2009. The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. *Diabetologia,* 52**,** 1766-1777.

D'AGOSTINO SR, R. B., VASAN, R. S., PENCINA, M. J., WOLF, P. A., COBAIN, M., MASSARO, J. M. & KANNEL, W. B. 2008. General cardiovascular risk profile for use in primary care: The Framingham heart study. *Circulation,* 117**,** 743-753.

DABELEA, D. 2007. The Predisposition to Obesity and Diabetes in Offspring of Diabetic Mothers. *Diabetes Care,* 30**,** S169-S174.

DANAEI, G., FINUCANE, M. M., LU, Y., SINGH, G. M., COWAN, M. J., PACIOREK, C. J., LIN, J. K., FARZADFAR, F., KHANG, Y. H., STEVENS, G. A., RAO, M., ALI, M. K., RILEY, L. M., ROBINSON, C. A. & EZZATI, M. 2011. National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: Systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants. *The Lancet,* 378**,** 31-40.

DE ONIS, M., BLÖSSNER, M. & BORGHI, E. 2010. Global prevalence and trends of overweight and obesity among preschool children. *The American Journal of Clinical Nutrition,* 92**,** 1257-1264.

DENLEY, A., WALLACE, J. C., COSGROVE, L. J. & FORBES, B. E. 2003. The Insulin Receptor Isoform Exon 11- (IR-A) in Cancer and Other Diseases: A Review. *Hormone and Metabolic Research,* 35**,** 778-785.

DRANOFF, G. 2004. Cytokines in cancer pathogenesis and cancer therapy. *Nature Reviews Cancer,* 4**,** 11-22.

DU, X. L., EDELSTEIN, D., DIMMELER, S., JU, Q., SUI, C. & BROWNLEE, M. 2001. Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *Journal of Clinical Investigation,* 108**,** 1341-1348.

EL-SERAG, H. B., HAMPEL, H. & JAVADI, F. 2006. The association between diabetes and hepatocellular carcinoma: A systematic review of epidemiologic evidence. *Clinical Gastroenterology and Hepatology,* 4**,** 369-380.

ELASHOFF, M., MATVEYENKO, A. V., GIER, B., ELASHOFF, R. & BUTLER, P. C. 2011. Pancreatitis, pancreatic, and thyroid cancer with Glucagon-like peptide-1based therapies. *Gastroenterology,* 141**,** 150-156.

FENECH, M. 2007. Cytokinesis-block micronucleus cytome assay. *Nature Protocols,* 2**,** 1084-1104.

FENECH, M. 2012. Folate (vitamin B9) and vitamin B12 and their function in the maintenance of nuclear and mitochondrial genome integrity. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis,* 733**,** 21-33.

FENECH, M., AITKEN, C. & RINALDI, J. 1998. Folate, vitamin B12, homocysteine status and DNA damage in young Australian adults. *Carcinogenesis,* 19**,** 1163-1171.

FENECH, M., BAGHURST, P., LUDERER, W., TURNER, J., RECORD, S., CEPPI, M. & BONASSI, S. 2005. Low intake of calcium, folate, nicotinic acid, vitamin E, retinol, β-carotene and high intake of pantothenic acid, biotin and riboflavin are significantly associated with increased

genome instability - Results from a dietary intake and micronucleus index survey in South Australia. *Carcinogenesis,* 26**,** 991-999.

FENECH, M., CHANG, W. P., KIRSCH-VOLDERS, M., HOLLAND, N., BONASSI, S. & ZEIGER, E. 2003. HUMN project: Detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis,* 534**,** 65-75.

FIGUEROA-ROMERO, C., SADIDI, M. & FELDMAN, E. L. 2008. Mechanisms of disease: The oxidative stress theory of diabetic neuropathy. *Reviews in Endocrine and Metabolic Disorders,* 9**,** 301-314.

FINKEL, T. & HOLBROOK, N. J. 2000. Oxidants, oxidative stress and the biology of ageing. *Nature,* 408**,** 239-247.

FIRTH, S. M. & BAXTER, R. C. 2002. Cellular Actions of the Insulin-Like Growth Factor Binding Proteins. *Endocrine Reviews,* 23**,** 824-854.

FORTE, V., PANDEY, A., ABDELMESSIH, R., FORTE, G., WHALEY-CONNELL, A., SOWERS, J. R. & MCFARLANE, S. I. 2012. Obesity, Diabetes, the Cardiorenal Syndrome, and Risk for Cancer. *Cardiorenal Medicine,* 2**,** 143-162.

FOWKE, J. H., MATTHEWS, C. M., BUCHOWSKI, M. S., SIGNORELLO, L. B., CHANG, S. S., COOKSON, M. S. & BLOT, W. J. 2008. Association between prostate-specific antigen and leptin, adiponectin, HbA1c or C-peptide among African-American and Caucasian men. *Prostate Cancer and Prostatic Diseases,* 11**,** 264-269.

FOWLER, M. J. 2011. Microvascular and macrovascular complications of diabetes. *Clinical Diabetes,* 29**,** 116-122.

FRIBERG, E., ORSINI, N., MANTZOROS, C. S. & WOLK, A. 2007. Diabetes mellitus and risk of endometrial cancer: A meta-analysis. *Diabetologia,* 50**,** 1365-1374.

FÜLÖP, N., MARCHASE, R. B. & CHATHAM, J. C. 2007. Role of protein O-linked N-acetyl-glucosamine in mediating cell function and survival in the cardiovascular system. *Cardiovascular Research,* 73**,** 288-297.

GABBAY, K. H. 1973. The sorbitol pathway and the complications of diabetes. *New England Journal of Medicine,* 288**,** 831-836.

GAZIEV, A. I., SOLOGUB, G. R., FOMENKO, L. A., ZAICHKINA, S. I., KOSYAKOVA, N. I. & BRADBURY, R. J. 1996. Effect of vitamin-antioxidant micronutrients on the frequency of spontaneous and in vitro γ-ray-induced micronuclei in lymphocytes of donors: The age factor. *Carcinogenesis,* 17**,** 493-499.

GERSTEIN, H. C., BOSCH, J., DAGENAIS, G. R., DÍAZ, R., JUNG, H., MAGGIONI, A. P., POGUE, J., PROBSTFIELD, J., RAMACHANDRAN, A., RIDDLE, M. C., RYDÉN, L. E. & YUSUF, S. 2012. Basal insulin and cardiovascular and other outcomes in dysglycemia. *New England Journal of Medicine,* 367**,** 319-328.

GIACCO, F. & BROWNLEE, M. 2010. Oxidative stress and diabetic complications. *Circulation Research,* 107**,** 1058-1070.

GIOVANNUCCI, E., HARLAN, D. M., ARCHER, M. C., BERGENSTAL, R. M., GAPSTUR, S. M., HABEL, L. A., POLLAK, M., REGENSTEINER, J. G. & YEE, D. 2010. Diabetes and cancer: A consensus report. *Diabetes Care,* 33**,** 1674-1685.

GOVINDARAJAN, R., RATNASINGHE, L., SIMMONS, D. L., SIEGEL, E. R., MIDATHADA, M. V., KIM, L., KIM, P. J., OWENS, R. J. & LANG, N. P. 2007. Thiazolidinediones and the risk of lung, prostate, and colon cancer in patients with diabetes. *Journal of Clinical Oncology,* 25**,** 1476-1481.

GUGLIUCCI, A. 2000. Glycation as the glucose link to diabetic complications. *Journal of the American Osteopathic Association,* 100**,** 621-634.

HAFER-MACKO, C. E., IVEY, F. M., SORKIN, J. D. & MACKO, R. F. 2007. Microvascular tissue plasminogen activator is reduced in diabetic neuropathy. *Neurology,* 69**,** 268-274.

HÄRING, H. U., KELLERER, M. & MOSTHAF, L. 1994. Modulation of insulin receptor signalling: Significance of altered receptor isoform patterns and mechanism of hyperglycaemia-induced receptor modulation. *Diabetologia,* 37**,** S149-S154.

HEMKENS, L. G., GROUVEN, U., BENDER, R., GÜNSTER, C., GUTSCHMIDT, S., SELKE, G. W. & SAWICKI, P. T. 2009. Risk of malignancies in patients with diabetes treated with human insulin or insulin analogues: A cohort study. *Diabetologia,* 52**,** 1732-1744.

HENRIKSEN, T., HILLESTRØM, P. R., POULSEN, H. E. & WEIMANN, A. 2009. Automated method for the direct analysis of 8-oxo-guanosine and 8-oxo-2′-deoxyguanosine in human urine using ultraperformance liquid chromatography and tandem mass spectrometry. *Free Radical Biology and Medicine,* 47**,** 629-635.

HOELZL, C., GLATT, H., MEINL, W., SONTAG, G., HAIDINGER, G., KUNDI, M., SIMIC, T., CHAKRABORTY, A., BICHLER, J., FERK, F., ANGELIS, K., NERSESYAN, A. & KNASMÜLLER, S. 2008. Consumption of Brussels sprouts protects peripheral human lymphocytes against 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhiP) and oxidative DNA-damage: Results of a controlled human intervention trial. *Molecular Nutrition and Food Research,* 52**,** 330-341.

HU, F. B. 2011. Globalization of diabetes: The role of diet, lifestyle, and genes. *Diabetes Care,* 34**,** 1249-1257.

HU, G., JOUSILAHTI, P., QIAO, Q., KATOH, S. & TUOMILEHTO, J. 2005. Sex differences in cardiovascular and total mortality among diabetic and non-diabetic individuals with or without history of myocardial infarction. *Diabetologia,* 48**,** 856-861.

HU, G., QIAO, Q. & TUOMILEHTO, J. 2001. Glucose tolerance and cardiovascular mortality. *Cardiovascular Reviews and Reports,* 22**,** 649-654+692.

HUXLEY, R., ANSARY-MOGHADDAM, A., BERRINGTON DE GONZÁLEZ, A., BARZI, F. & WOODWARD, M. 2005. Type-II diabetes and pancreatic cancer: A meta-analysis of 36 studies. *British Journal of Cancer,* 92**,** 2076-2083.

IBARRA-COSTILLA, E., CERDA-FLORES, R. M., DÁVILA-RODRÍGUEZ, M. I., SAMAYO-REYES, A., CALZADO-FLORES, C. & CORTÉS-GUTIÉRREZ, E. I. 2010. DNA damage evaluated by comet assay in Mexican patients with type 2 diabetes mellitus. *Acta Diabetologica,* 47**,** S111-S116.

INTERNATIONAL DIABETES FEDERATION. 2012. IDF Diabetes Atlas. 5th edn. Brussels, Belgium: Internationals Daibetes Federation. http//www.idf.org/diabetesatlas (22.02.2013)

JAISWAL, M., LARUSSO, N. F., BURGART, L. J. & GORES, G. J. 2000. Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. *Cancer Research,* 60**,** 184-190.

JAKOB, E. & ELMADFA, I. 1995. Rapid HPLC assay for the assessment of vitamin K1, A, E and beta-carotene status in children (7-19 years). *International Journal for Vitamin and Nutrition Research,* 65**,** 31-35.

JIANG, Y., BEN, Q., SHEN, H., LU, W., ZHANG, Y. & ZHU, J. 2011. Diabetes mellitus and incidence and mortality of colorectal cancer: A systematic review and meta-analysis of cohort studies. *European Journal of Epidemiology,* 26**,** 863-876.

JOHNSON, J. A., CARSTENSEN, B., WITTE, D., BOWKER, S. L., LIPSCOMBE, L. & RENEHAN, A. G. 2012. Diabetes and cancer (1): Evaluating the temporal relationship between type 2 diabetes and cancer incidence. *Diabetologia,* 55**,** 1607-1618.

JONASSON, J. M., LJUNG, R., TALBÄCK, M., HAGLUND, B., GUDBJÖRNSDÒTTIR, S. & STEINECK, G. 2009. Insulin glargine use and short-term incidence of malignancies-a population-based follow-up study in Sweden. *Diabetologia,* 52**,** 1745-1754.

JOSHU, C. E., PRIZMENT, A. E., DLUZNIEWSKI, P. J., MENKE, A., FOLSOM, A. R., CORESH, J., YEH, H. C., BRANCATI, F. L., PLATZ, E. A. & SELVIN, E. 2012. Glycated hemoglobin and cancer

incidence and mortality in the Atherosclerosis in Communities (ARIC) Study, 1990-2006. *International Journal of Cancer,* 131**,** 1667-1677.

JOUSSEN, A. M., POULAKI, V., LE, M. L., KOIZUMI, K., ESSER, C., JANICKI, H., SCHRAERMEYER, U., KOCIOK, N., FAUSER, S., KIRCHHOF, B., KERN, T. S. & ADAMIS, A. P. 2004. A central role for inflammation in the pathogenesis of diabetic retinopathy. *FASEB Journal,* 18**,** 1450-1452.

KAAKS, R. & LUKANOVA, A. 2001. Energy balance and cancer: The role of insulin and insulin-like growth factor-I. *Proceedings of the Nutrition Society,* 60**,** 91-106.

KAAKS, R., LUKANOVA, A. & KURZER, M. S. 2002. Obesity, endogenous hormones, and endometrial cancer risk: A synthetic review. *Cancer Epidemiology Biomarkers and Prevention,* 11**,** 1531-1543.

KAHN, S. E., HULL, R. L. & UTZSCHNEIDER, K. M. 2006. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature,* 444**,** 840-846.

KANWAR, Y. S., WADA, J., SUN, L., XIE, P., WALLNER, E. I., CHEN, S., CHUGH, S. & DANESH, F. R. 2008. Diabetic nephropathy: Mechanisms of renal disease progression. *Experimental Biology and Medicine,* 233**,** 4-11.

KASPER, J. S. & GIOVANNUCCI, E. 2006. A meta-analysis of diabetes mellitus and the risk of prostate cancer. *Cancer Epidemiology Biomarkers and Prevention,* 15**,** 2056-2062.

KEOGH, R. J., DUNLOP, M. E. & LARKINS, R. G. 1997. Effect of inhibition of aldose reductase on glucose flux, diacylglycerol formation, protein kinase C, and phospholipase A2 activation. *Metabolism: Clinical and Experimental,* 46**,** 41-47.

KEY, T. J., APPLEBY, P. N., REEVES, G. K., RODDAM, A., DORGAN, J. F., LONGCOPE, C., STANCZYK, F. Z., STEPHENSON JR, H. E., FALK, R. T., MILLER, R., SCHATZKIN, A., ALLEN, D. S., FENTIMAN, I. S., WANG, D. Y., DOWSETT, M., THOMAS, H. V., HANKINSON, S. E., TONIOLO, P., AKHMEDKHANOV, A., KOENIG, K., SHORE, R. E., ZELENIUCH-JACQUOTTE, A., BERRINO, F., MUTI, P., MICHELI, A., KROGH, V., SIERI, S., PALA, V., VENTURELLI, E., SECRETO, G., BARRETT-CONNOR, E., LAUGHLIN, G. A., KABUTO, M., AKIBA, S., STEVENS, R. G., NERIISHI, K., LAND, C. E., CAULEY, J. A., KULLER, L. H., CUMMINGS, S. R., HELZLSOUER, K. J., ALBERG, A. J., BUSH, T. L., COMSTOCK, G. W., GORDON, G. B. & MILLER, S. R. 2003. Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. *Journal of the National Cancer Institute,* 95**,** 1218-1226.

KHANDWALA, H. M., MCCUTCHEON, I. E., FLYVBJERG, A. & FRIEND, K. E. 2000. The Effects of Insulin-Like Growth Factors on Tumorigenesis and Neoplastic Growth. *Endocrine Reviews,* 21**,** 215-244.

KING, G. L. & LOEKEN, M. R. 2004. Hyperglycemia-induced oxidative stress in diabetic complications. *Histochemistry and Cell Biology,* 122**,** 333-338.

KNUDSEN, L. B., MADSEN, L. W., ANDERSEN, S., ALMHOLT, K., DE BOER, A. S., DRUCKER, D. J., GOTFREDSEN, C., EGEROD, F. L., HEGELUND, A. C., JACOBSEN, H., JACOBSEN, S. D., MOSES, A. C., MØLCK, A. M., NIELSEN, H. S., NOWAK, J., SOLBERG, H., THI, T. D. L. & ZDRAVKOVIC, M. 2010. Glucagon-like peptide-1 receptor agonists activate rodent thyroid C-cells causing calcitonin release and C-cell proliferation. *Endocrinology,* 151**,** 1473-1486.

KOLB, H. & MANDRUP-POULSEN, T. 2010. The global diabetes epidemic as a consequence of lifestyle-induced low-grade inflammation. *Diabetologia,* 53**,** 10-20.

KORO, C., BARRETT, S. & QIZILBASH, N. 2007. Cancer risks in thiazolidinedione users compared to other anti-diabetic agents. *Pharmacoepidemiology and Drug Safety,* 16**,** 485-492.

KOYA, D. & KING, G. L. 1998. Protein kinase C activation and the development of diabetic complications. *Diabetes,* 47**,** 859-866.

LANDMAN, G. W. D., KLEEFSTRA, N., VAN HATEREN, K. J. J., GROENIER, K. H., GANS, R. O. B. & BILO, H. J. G. 2010. Metformin associated with lower cancer mortality in type 2 diabetes: Zodiac-16. *Diabetes Care,* 33**,** 322-326.

LARSSON, S. C., ORSINI, N., BRISMAR, K. & WOLK, A. 2006. Diabetes mellitus and risk of bladder cancer: A meta-analysis. *Diabetologia,* 49**,** 2819-2823.

LARSSON, S. C., ORSINI, N. & WOLK, A. 2005. Diabetes mellitus and risk of colorectal cancer: A meta-analysis. *Journal of the National Cancer Institute,* 97**,** 1679-1687.

LARSSON, S. C. & WOLK, A. 2011. Diabetes mellitus and incidence of kidney cancer: A meta-analysis of cohort studies. *Diabetologia,* 54**,** 1013-1018.

LAW, J. H., HABIBI, G., HU, K., MASOUDI, H., WANG, M. Y. C., STRATFORD, A. L., PARK, E., GEE, J. M. W., FINLAY, P., JONES, H. E., NICHOLSON, R. I., CARBONI, J., GOTTARDIS, M., POLLAK, M. & DUNN, S. E. 2008. Phosphorylated insulin-like growth factor-I/insulin receptor is present in all breast cancer subtypes and is related to poor survival. *Cancer Research,* 68**,** 10238-10246.

LAZALDE-RAMOS, B. P., ZAMORA-PEREZ, A. L., SOSA-MACÍAS, M., GUERRERO-VELÁZQUEZ, C. & ZÚÑIGA-GONZÁLEZ, G. M. 2012. DNA and Oxidative Damages Decrease After Ingestion of Folic Acid in Patients with Type 2 Diabetes. *Archives of Medical Research,* 43**,** 476-481.

LEAHY, J. L. 2005. Pathogenesis of type 2 diabetes mellitus. *Archives of Medical Research,* 36**,** 197-209.

LEAN, M. E. J., NOROOZI, M., KELLY, I., BURNS, J., TALWAR, D., SATTAR, N. & CROZIER, A. 1999. Dietary flavonols protect diabetic human lymphocytes against oxidative damage to DNA. *Diabetes,* 48**,** 176-181.

LEVIN, E. R. 2002. Cellular functions of plasma membrane estrogen receptors. *Steroids,* 67**,** 471-475.

LIBBY, G., DONNELLY, L. A., DONNAN, P. T., ALESSI, D. R., MORRIS, A. D. & EVANS, J. M. M. 2009. New users of metformin are at low risk of incident cancer: A cohort study among people with type 2 diabetes. *Diabetes Care,* 32**,** 1620-1625.

LODOVICI, M., GIOVANNELLI, L., PITOZZI, V., BIGAGLI, E., BARDINI, G. & ROTELLA, C. M. 2008. Oxidative DNA damage and plasma antioxidant capacity in type 2 diabetic patients with good and poor glycaemic control. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis,* 638**,** 98-102.

LUO, W., CAO, Y., LIAO, C. & GAO, F. 2012. Diabetes mellitus and the incidence and mortality of colorectal cancer: A meta-analysis of 24 cohort studies. *Colorectal Disease,* 14**,** 1307-1312.

MAEDA, N., SHIMOMURA, I., KISHIDA, K., NISHIZAWA, H., MATSUDA, M., NAGARETANI, H., FURUYAMA, N., KONDO, H., TAKAHASHI, M., ARITA, Y., KOMURO, R., OUCHI, N., KIHARA, S., TOCHINO, Y., OKUTOMI, K., HORIE, M., TAKEDA, S., AOYAMA, T., FUNAHASHI, T. & MATSUZAWA, Y. 2002. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med,* 8**,** 731-737.

MAJCHRZAK, D., SINGER, I., MÄNNER, M., RUST, P., GENSER, D., WAGNER, K. H. & ELMADFA, I. 2007. B-vitamin status and concentrations of homocysteine in Austrian omnivores, vegetarians and vegans. *Annals of Nutrition and Metabolism,* 50**,** 485-491.

MANTOVANI, A., ALLAVENA, P., SICA, A. & BALKWILL, F. 2008. Cancer-related inflammation. *Nature,* 454**,** 436-444.

MARSHALL, S., BACOTE, V. & TRAXINGER, R. R. 1991. Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system: role of hexosamine in the induction of insulin resistance. *Journal of Biological Chemistry,* 266**,** 4706-4712.

MARTÍNEZ-PÉREZ, L. M., CERDA-FLORES, R. M., GALLEGOS-CABRIALES, E. C., DÁVILA-RODRÍGUEZ, M. I., IBARRA-COSTILLA, E. & CORTÉS-GUTIÉRREZ, E. I. 2007. Frequency of micronuclei in Mexicans with type 2 diabetes mellitus. *Prague medical report,* 108**,** 248-255.

MASSÓ GONZÁLEZ, E. L., JOHANSSON, S., WALLANDER, M. A. & GARCÍA RODRÍGUEZ, L. A. 2009. Trends in the prevalence and incidence of diabetes in the UK: 1996-2005. *Journal of Epidemiology and Community Health,* 63**,** 332-336.

MATTHEWS, D. R., HOSKER, J. P. & RUDENSKI, A. S. 1985. Homeostasis model assessment: Insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia,* 28**,** 412-419.

MCGARRY, J. D. & DOBBINS, R. L. 1999. Fatty acids, lipotoxicity and insulin secretion. *Diabetologia,* 42**,** 128-138.

MERCER, J. R., CHENG, K. K., FIGG, N., GORENNE, I., MAHMOUDI, M., GRIFFIN, J., VIDAL-PUIG, A., LOGAN, A., MURPHY, M. P. & BENNETT, M. 2010. DNA damage links mitochondrial dysfunction to atherosclerosis and the metabolic syndrome. *Circulation Research,* 107**,** 1021-1031.

MEYER, K. A., KUSHI, L. H., JACOBS JR, D. R. & FOLSOM, A. R. 2001. Dietary fat and incidence of type 2 diabetes in older Iowa women. *Diabetes Care,* 24**,** 1528-1535.

MIAO JONASSON, J., CEDERHOLM, J., ELIASSON, B., ZETHELIUS, B., EEG-OLOFSSON, K. & GUDBJÖRNSDOTTIR, S. 2012. HbA1C and cancer risk in patients with type 2 diabetes - a nationwide population-based prospective cohort study in Sweden. *PLoS ONE,* 7.

MITRI, J., CASTILLO, J. & PITTAS, A. G. 2008. Diabetes and risk of non-hodgkin's lymphoma: A meta-analysis of observational studies. *Diabetes Care,* 31**,** 2391-2397.

MIYAKE, H., HARA, I., KAMIDONO, S. & ETO, H. 2004. Oxidative DNA damage in patients with prostate cancer and its response to treatment. *Journal of Urology,* 171**,** 1533-1536.

MØLLER, P., VOGEL, U., PEDERSEN, A., DRAGSTED, L. O., SANDSTRÖM, B. & LOFT, S. 2003. No Effect of 600 Grams Fruit and Vegetables Per Day on Oxidative DNA Damage and Repair in Healthy Nonsmokers. *Cancer Epidemiology Biomarkers and Prevention,* 12**,** 1016-1022.

MOLNÁR, D. 2004. The prevalence of the metabolic syndrome and type 2 diabetes mellitus in children and adolescents. *International Journal of Obesity,* 28**,** S70-S74.

MONAMI, M., LAMANNA, C., BALZI, D., MARCHIONNI, N. & MANNUCCI, E. 2009. Sulphonylureas and cancer: A case-control study. *Acta Diabetologica,* 46**,** 279-284.

MONAMI, M., LAMANNA, C., MARCHIONNI, N. & MANNUCCI, E. 2008. Rosiglitazone and risk of cancer: A meta-analysis of randomized clinical trials. *Diabetes Care,* 31**,** 1455-1460.

MOSER, B., SZEKERES, T., BIEGLMAYER, C., WAGNER, K. H., MIŠÍK, M., KUNDI, M., ZAKERSKA, O., NERSESYAN, A., KAGER, N., ZAHRL, J., HOELZL, C., EHRLICH, V. & KNASMUELLER, S. 2011. Impact of spinach consumption on DNA stability in peripheral lymphocytes and on biochemical blood parameters: Results of a human intervention trial. *European Journal of Nutrition,* 50**,** 587-594.

MURGIA, E., BALLARDIN, M., BONASSI, S., ROSSI, A. M. & BARALE, R. 2008. Validation of micronuclei frequency in peripheral blood lymphocytes as early cancer risk biomarker in a nested case–control study. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 639**,** 27-34.

MYNARCIK, D. C., WILLIAMS, P. F., SCHAFFER, L., YU, G. Q. & WHITTAKER, J. 1997. Identification of common ligand binding determinants of the insulin and insulin-like growth factor 1 receptors. Insights into mechanisms of ligand binding. *Journal of Biological Chemistry,* 272**,** 18650-18655.

NATHAN, D. M., BALKAU, B., BONORA, E., BORCH-JOHNSEN, K., BUSE, J. B., COLAGIURI, S., DAVIDSON, M. B., DEFRONZO, R., GENUTH, S., HOLMAN, R. R., JI, L., KIRKMAN, S., KNOWLER, W. C., SCHATZ, D., SHAW, J., SOBNGWI, E., STEFFES, M., VACCARO, O., WAREHAM, N., ZINMAN, B. & KAHN, R. 2009a. International expert committee report on the role of the A1C assay in the diagnosis of diabetes. *Diabetes Care,* 32**,** 1327-1334.

NATHAN, D. M., BUSE, J. B., DAVIDSON, M. B., FERRANNINI, E., HOLMAN, R. R., SHERWIN, R. & ZINMAN, B. 2009b. Medical management of hyperglycemia in type 2 diabetes: A consensus algorithm for the initiation and adjustment of therapy. *Diabetes Care,* 32**,** 193-203.

NESTLER, J. E. 1997. Insulin regulation of human ovarian androgens. *Human reproduction (Oxford, England),* 12 Suppl 1**,** 53-62.

NISHIKAWA, T., EDELSTEIN, D., DU, X. L., YAMAGISHI, S. I., MATSUMURA, T., KANEDA, Y., YOREK, M. A., BEEBE, D., OATES, P. J., HAMMES, H. P., GLARDINO, I. & BROWNLEE, M. 2000. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature,* 404**,** 787-790.

NOLAN, C. J., DAMM, P. & PRENTKI, M. 2011. Type 2 diabetes across generations: From pathophysiology to prevention and management. *The Lancet,* 378**,** 169-181.

NUSSLER, A. K., GELLER, D. A., SWEETLAND, M. A., DI SILVIO, M., BILLIAR, T. R., MADARIAGA, J. B., SIMMONS, R. L. & LANCASTER JR, J. R. 1993. Induction of nitric oxide synthesis and its reactions in cultured human and rat hepatocytes stimulated with cystokines plus LPS. *Biochemical and Biophysical Research Communications,* 194**,** 826-835.

OATES, P. J. 2008. Aldose reductase, still a compelling target for diabetic neuropathy. *Current Drug Targets,* 9**,** 14-36.

OHWAKI, K., ENDO, F., MURAISHI, O. & YANO, E. 2011. Relationship between changes in haemoglobin A 1C and prostate-specific antigen in healthy men. *European Journal of Cancer,* 47**,** 262-266.

PAPA, V., PEZZINO, V., COSTANTINO, A., BELFIORE, A., GIUFFRIDA, D., FRITTITTA, L., VANNELLI, G. B., BRAND, R., GOLDFINE, I. D. & VIGNERI, R. 1990. Elevated insulin receptor content in human breast cancer. *Journal of Clinical Investigation,* 86**,** 1503-1510.

PIIRONEN, V., TOIVO, J. & LAMPI, A. M. 2002. New data for cholesterol contents in meat, fish, milk, eggs and their products consumed in Finland. *Journal of Food Composition and Analysis,* 15**,** 705-713.

PIÑERO-PILOÑA, A. & RASKIN, P. 2001. Idiopathic Type 1 diabetes. *Journal of Diabetes and its Complications,* 15**,** 328-335.

PITOZZI, V., GIOVANNELLI, L., BARDINI, G., ROTELLA, C. M. & DOLARA, P. 2003. Oxidative DNA damage in peripheral blood cells in type 2 diabetes mellitus: Higher vulnerability of polymorphonuclear leukocytes. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis,* 529**,** 129-133.

POLET, F. & FERON, O. 2013. Endothelial cell metabolism and tumour angiogenesis: Glucose and glutamine as essential fuels and lactate as the driving force. *Journal of Internal Medicine,* 273**,** 156-165.

POLLARD, J. W. 2004. Tumour-educated macrophages promote tumour progression and metastasis. *Nature Reviews Cancer,* 4**,** 71-78.

POULSEN, H. E. 2005. Oxidative DNA modifications. *Experimental and Toxicologic Pathology,* 57**,** 161-169.

POULSEN, H. E., SPECHT, E., BROEDBAEK, K., HENRIKSEN, T., ELLERVIK, C., MANDRUP-POULSEN, T., TONNESEN, M., NIELSEN, P. E., ANDERSEN, H. U. & WEIMANN, A. 2012. RNA modifications by oxidation: A novel disease mechanism? *Free Radical Biology and Medicine,* 52**,** 1353-1361.

RAMOS-NINO, M. E., MACLEAN, C. D. & LITTENBERG, B. 2007. Association between cancer prevalence and use of thiazolidinediones: Results from the Vermont Diabetes Information System. *BMC Medicine,* 5.

RAPP, K., SCHROEDER, J., KLENK, J., ULMER, H., CONCIN, H., DIEM, G., OBERAIGNER, W. & WEILAND, S. K. 2006. Fasting blood glucose and cancer risk in a cohort of more than 140,000 adults in Austria. *Diabetologia,* 49**,** 945-952.

RISO, P., VISIOLI, F., GRANDE, S., GUARNIERI, S., GARDANA, C., SIMONETTI, P. & PORRINI, M. 2006. Effect of a tomato-based drink on markers of inflammation, immunomodulation, and oxidative stress. *Journal of Agricultural and Food Chemistry,* 54**,** 2563-2566.

ROBERTS, D. L., DIVE, C. & RENEHAN, A. G. 2010. Biological mechanisms linking obesity and cancer risk: New perspectives. *Annual Reviews of Medicine,* 61**,** 301-316.

SALMERÓN, J., HU, F. B., MANSON, J. E., STAMPFER, M. J., COLDITZ, G. A., RIMM, E. B. & WILLETT, W. C. 2001. Dietary fat intake and risk of type 2 diabetes in women. *American Journal of Clinical Nutrition,* 73**,** 1019-1026.

SALTIEL, A. R. & KAHN, C. R. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature,* 414**,** 799-806.

SANDHU, M. S., DUNGER, D. B. & GIOVANNUCCI, E. L. 2002. Insulin, insulin-like growth factor-I (IGF-I), IGF binding proteins, their biologic interactions, and colorectal cancer. *Journal of the National Cancer Institute,* 94**,** 972-980.

ŞARDAŞ, S., YILMAZ, M., ÖZTOK, U., ÇAKIR, N. & KARAKAYA, A. E. 2001. Assessment of DNA strand breakage by comet assay in diabetic patients and the role of antioxidant supplementation. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis,* 490**,** 123-129.

SATOH, J., YAGIHASHI, S. & TOYOTA, T. 2003. The possible role of tumor necrosis factor-α in diabetic polyneuropathy. *Experimental Diabesity Research,* 4**,** 65-71.

SCHMIDT, A. M., YAN, S. D., WAUTIER, J.-L. & STERN, D. 1999. Activation of Receptor for Advanced Glycation End Products. *Circulation Research,* 84**,** 489-497.

SCHNEIDER, M., DIEMER, K., ENGELHART, K., ZANKL, H., TROMMER, W. E. & BIESALSKI, H. K. 2001. Protective effects of vitamins C and E on the numbers of micronuclei in lymphocytes in smokers and their role in ascorbate free radical formation in plasma. *Free Radical Research,* 34**,** 209-219.

SCHOFIELD, C. J. & SUTHERLAND, C. 2012. Disordered insulin secretion in the development of insulin resistance and Type 2 diabetes. *Diabetic Medicine,* 29**,** 972-979.

SCHREIBER, V., DANTZER, F., AMÉ, J. C. & DE MURCIA, G. 2006. Poly(ADP-ribose): Novel functions for an old molecule. *Nature Reviews Molecular Cell Biology,* 7**,** 517-528.

SESHASAI, S. R. K., KAPTOGE, S., THOMPSON, A., DI ANGELANTONIO, E., GAO, P., SARWAR, N., WHINCUP, P. H., MUKAMAL, K. J., GILLUM, R. F., HOLME, I., NJØLSTAD, I., FLETCHER, A., NILSSON, P., LEWINGTON, S., COLLINS, R., GUDNASON, V., THOMPSON, S. G., SATTAR, N.,

SELVIN, E., HU, F. B. & DANESH, J. 2011. Diabetes mellitus, fasting glucose, and risk of cause-specific death. *New England Journal of Medicine,* 364**,** 829-841.

SHETTIGAR, S. K. G., SHAILAJA, C. & KULKARNI, R. K. 2012. Elevated micronuclei frequency in type 2 diabetes with high glycosylated hemoglobin. *Diabetes Research and Clinical Practice,* 95**,** 246-250.

SMITH, U. & GALE, E. A. M. 2009. Editorial: Does diabetes therapy influence the risk of cancer? *Diabetologia,* 52**,** 1699-1708.

SMOLKOVÁ, B., DUŠINSKÁ, M., RAŠLOVÁ, K., BARANČOKOVÁ, M., KAŽIMÍROVÁ, A., HORSKÁ, A., SPUSTOVÁ, V. & COLLINS, A. 2004. Folate levels determine effect of antioxidant supplementation on micronuclei in subjects with cardiovascular risk. *Mutagenesis,* 19**,** 469-476.

SONG, F., JIA, W., YAO, Y., HU, Y., LEI, L., LIN, J., SUN, X. & LIU, L. 2007. Oxidative stress, antioxidant status and DNA damage in patients with impaired glucose regulation and newly diagnosed type 2 diabetes. *Clinical Science,* 112**,** 599-606.

SOOS, M. A., FIELD, C. E. & SIDDLE, K. 1993. Purified hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I, but not insulin, with high affinity. *Biochemical Journal,* 290**,** 419-426.

STAMLER, J., VACCARO, O., NEATON, J. D. & WENTWORTH, D. 1993. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the multiple risk factor intervention trial. *Diabetes Care,* 16**,** 434-444.

STEVENS, R. J., ALI, R., BANKHEAD, C. R., BETHEL, M. A., CAIRNS, B. J., CAMISASCA, R. P., CROWE, F. L., FARMER, A. J., HARRISON, S., HIRST, J. A., HOME, P., KAHN, S. E., MCLELLAN, J. H., PERERA, R., PLÜDDEMANN, A., RAMACHANDRAN, A., ROBERTS, N. W., ROSE, P. W., SCHWEIZER, A., VIBERTI, G. & HOLMAN, R. R. 2012. Cancer outcomes and all-cause mortality in adults allocated to metformin: Systematic review and collaborative meta-analysis of randomised clinical trials. *Diabetologia,* 55**,** 2593-2603.

STRATTON, M. R., CAMPBELL, P. J. & FUTREAL, P. A. 2009. The cancer genome. *Nature,* 458**,** 719-724.

STUMVOLL, M., GOLDSTEIN, B. J. & VAN HAEFTEN, T. W. 2005. Type 2 diabetes: principles of pathogenesis and therapy. *The Lancet,* 365**,** 1333-1346.

SZABO, C. 2009. Role of nitrosative stress in the pathogenesis of diabetic vascular dysfunction: REVIEW. *British Journal of Pharmacology,* 156**,** 713-727.

TABÁK, A. G., HERDER, C., RATHMANN, W., BRUNNER, E. J. & KIVIMÄKI, M. 2012. Prediabetes: a high-risk state for diabetes development. *The Lancet,* 379**,** 2279-2290.

TAGESSON, C., KALLBERG, M., KLINTENBERG, C. & STARKHAMMAR, H. 1995. Determination of urinary 8-hydroxydeoxyguanosine by automated coupled-column high performance liquid chromatography: A powerful technique for assaying in vivo oxidative DNA damage in cancer patients. *European Journal of Cancer Part A: General Topics,* 31**,** 934-940.

TAHRANI, A. A., BAILEY, C. J., DEL PRATO, S. & BARNETT, A. H. 2011. Management of type 2 diabetes: New and future developments in treatment. *The Lancet,* 378**,** 182-197.

THANAN, R., MURATA, M., PINLAOR, S., SITHITHAWORN, P., KHUNTIKEO, N., TANGKANAKUL, W., HIRAKU, Y., OIKAWA, S., YONGVANIT, P. & KAWANISHI, S. 2008. Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine in patients with parasite infection and effect of antiparasitic drug in relation to cholangiocarcinogenesis. *Cancer Epidemiology Biomarkers and Prevention,* 17**,** 518-524.

THOMAS, P., HOLLAND, N., BOLOGNESI, C., KIRSCH-VOLDERS, M., BONASSI, S., ZEIGER, E., KNASMUELLER, S. & FENECH, M. 2009. Buccal micronucleus cytome assay. *Nature Protocols,* 4**,** 825-837.

THOMAS, P., WU, J., DHILLON, V. & FENECH, M. 2011. Effect of dietary intervention on human micronucleus frequency in lymphocytes and buccal cells. *Mutagenesis,* 26**,** 69-76.

TILG, H. & MOSCHEN, A. R. 2006. Adipocytokines: Mediators linking adipose tissue, inflammation and immunity. *Nature Reviews Immunology,* 6**,** 772-783.

TITENKO-HOLLAND, N., JACOB, R. A., SHANG, N., BALARAMAN, A. & SMITH, M. T. 1998. Micronuclei in lymphocytes and exfoliated buccal cells of postmenopausal women with dietary changes in folate. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis,* 417**,** 101-114.

TRUMPOWER, B. L. 1990. The protonmotive Q cycle. Energy transduction by coupling of proton translocation to electron transfer by the cytochrome bc1 complex. *Journal of Biological Chemistry,* 265**,** 11409-11412.

VAN DEN BERG, R., VAN VLIET, T., BROEKMANS, W. M. R., CNUBBEN, N. H. P., VAES, W. H. J., ROZA, L., HAENEN, G. R. M. M., BAST, A. & VAN DEN BERG, H. 2001. A vegetable/fruit concentrate with high antioxidant capacity has no effect on biomarkers of antioxidant status in male smokers. *Journal of Nutrition,* 131**,** 1714-1722.

VAN DIEREN, S., BEULENS, J. W. J., VAN DER SCHOUW, Y. T., GROBBEE, D. E. & NEAL, B. 2010. The global burden of diabetes and its complications: An emerging pandemic. *European Journal of Cardiovascular Prevention and Rehabilitation,* 17**,** S3-S8.

VIGNERI, P., FRASCA, F., SCIACCA, L., PANDINI, G. & VIGNERI, R. 2009. Diabetes and cancer. *Endocrine-Related Cancer,* 16**,** 1103-1123.

WAGNER, K. H., AUER, E. & ELMADFA, I. 2000. Content of trans fatty acids in margarines, plant oils, fried products and chocolate spreads in Austria. *European Food Research and Technology,* 210**,** 237-241.

WANG, C., WANG, X., GONG, G., BEN, Q., QIU, W., CHEN, Y., LI, G. & WANG, L. 2012. Increased risk of hepatocellular carcinoma in patients with diabetes mellitus: A systematic review and meta-analysis of cohort studies. *International Journal of Cancer,* 130**,** 1639-1648.

WENG, J., LI, Y., XU, W., SHI, L., ZHANG, Q., ZHU, D., HU, Y., ZHOU, Z., YAN, X., TIAN, H., RAN, X., LUO, Z., XIAN, J., YAN, L., LI, F., ZENG, L., CHEN, Y., YANG, L., YAN, S., LIU, J., LI, M., FU, Z. & CHENG, H. 2008. Effect of intensive insulin therapy on β-cell function and glycaemic control in patients with newly diagnosed type 2 diabetes: a multicentre randomised parallel-group trial. *The Lancet,* 371**,** 1753-1760.

WHEELER, M. L., DUNBAR, S. A., JAACKS, L. M., KARMALLY, W., MAYER-DAVIS, E. J., WYLIE-ROSETT, J. & YANCY, W. S. 2012. Macronutrients, food groups, and eating patterns in the management of diabetes: A systematic review of the literature, 2010. *Diabetes Care,* 35**,** 1395.

WHITING, D. R., GUARIGUATA, L., WEIL, C. & SHAW, J. 2011. IDF Diabetes Atlas: Global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes Research and Clinical Practice,* 94**,** 311-321.

WRIGHT, J. L. & STANFORD, J. L. 2009. Metformin use and prostate cancer in Caucasian men: Results from a population-based case-control study. *Cancer Causes and Control,* 20**,** 1617-1622.

YU, H., PARDOLL, D. & JOVE, R. 2009. STATs in cancer inflammation and immunity: A leading role for STAT3. *Nature Reviews Cancer,* 9**,** 798-809.

YUHARA, H., STEINMAUS, C., COHEN, S. E., CORLEY, D. A., TEI, Y. & BUFFLER, P. A. 2011. Is diabetes mellitus an independent risk factor for colon cancer and rectal cancer. *American Journal of Gastroenterology,* 106**,** 1911-1921.

ZAKIKHANI, M., DOWLING, R., FANTUS, I. G., SONENBERG, N. & POLLAK, M. 2006. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. *Cancer Research,* 66**,** 10269-10273.

ZHANG, P., ZHANG, X., BROWN, J., VISTISEN, D., SICREE, R., SHAW, J. & NICHOLS, G. 2010. Global healthcare expenditure on diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice,* 87**,** 293-301.

ZÚÑIGA-GONZÁLEZ, G. M., BATISTA-GONZÁLEZ, C. M., GÓMEZ-MEDA, B. C., RAMOS-IBARRA, M. L., ZAMORA-PEREZ, A. L., MUÑOZ-MAGALLANES, T., RAMOS-VALDÉS, C. & GALLEGOS-ARREOLA, M. P. 2007. Micronuclei in diabetes: Folate supplementation diminishes micronuclei in diabetic patients but not in an animal model. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis,* 634**,** 126-134.

8 Acknowledgement

I'd like to thank everybody who contributed to this thesis, supported and encouraged me during the last few years. Special thanks to:

My supervisor Prof. Karl-Heinz Wagner for your support, your trust in my work, for being always positive, and creating a friendly, stimulating working atmosphere.

Prof. Ďuračková Zdeňka, professor at Comenius University in Bratislava, and your team for being part of this INTERREG project, with all its challenges in different aspects.

Dr. Helmut Brath, head of the diabetes outpatient clinic, for recruiting participants for the intervention study in spite of your tight schedule. Thanks for your valuable support and your interest in nutritional sciences.

Prof. Andrew Collins and Dr. Amaya Azqueta for your scientific advice, for sharing your knowledge about Comet Assay with me and answering patiently all me questions.

All former and present colleagues, diploma/master students and friends at the Department of Nutritional Sciences for your help, valuable advice, fruitful discussions, sharing all ups and downs and still having fun together.

My family and friends for being there for me and bringing a smile to my face.

علی عزیزم: مرسی از اینکه همیشه به یاد من هستی و من می توانم روی تو حساب کنم.

9 Funding

This work was funded by EU, through the cross-border cooperation programme Slovakia – Austria 2007-2013 (http://www.sk-at.eu) and the Austrian Ministry of Health.

I endeavoured to identify all image copyright holders to obtain their agreement to use their images in the present thesis. However, in case of any copyright infringement, I kindly request personal communication.

Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

Curriculum Vitae

Personal Information:

Name:	Elisabeth Müllner
Date of Birth:	June 3rd, 1983
Citizenship:	Austria

Current position:

Scientific assistant at the Department of Nutritional Sciences (prae-doc position), University of Vienna; lecturer at the Department of Nutritional Sciences

Education:

June 2001	High School Diploma at the Bundesgymnasium Horn
Oct 2001 – Jan 2008	Study of Nutritional Sciences, University of Vienna
	Title of master thesis: *„Veränderung der Konzentration fettlöslicher Vitamine und des Fettsäuremusters bei Typ-II-Diabetikern nach Pflanzenölintervention“*
	Supervisor: Univ. Prof. Dr. Karl-Heinz Wagner
Jan 2008	Graduated as Mag. rer. nat. with first class honours
Oct 2008 – May 2009	Scientific Co-worker at the Department of Nutritional Sciences
Since June 2009	PhD student at the Department of Nutritional Sciences
	Faculty of Life Sciences, University of Vienna
	Title of dissertation: *“Influence of a lifestyle intervention on oxidative stability in type II diabetes”*

Research activities abroad:

April – Sept 2008	Research visit in the working group of Prof. Jana Pickova, Swedish University of Agricultural Sciences, Department of Food Sciences, Uppsala, Sweden
Sept 2009	Research visit at the laboratory of Prof. Andrew R. Collins, Department of Nutrition, University of Oslo, Norway

Aug 2012	Research visit at the laboratory of Prof. Andrew R. Collins, Department of Nutrition, University of Oslo, Norway

Work Experience:

Feb 2004	Internship at the University of Vienna, Department of Nutritional Sciences
July 2005	Internship at a food production company in Liechtenstein
Sept 2005	Internship at the central medical laboratory in Feldkirch
July 2006	Internship at a dairy in Feldkirch
March – June 2009	Assistant lecturer at the Department of Behavioural Biology, University of Vienna
Oct 2009 – June 2010	Tutor at the Department of Nutritional Sciences
Since Nov 2010	Lecturer at the Department of Nutritional Sciences

Scholarships:

Jan 2009 – Sept 2009	Awarded with a research grant on behalf of the University of Vienna (Forschungsstipendium)

Professional Qualifications:

- Analytical skills: HPLC (vitamin analysis), GC (fatty acid analysis), cell cultures, comet assay, comet repair assay, cytokinesis-block micronucleus assay
- Designing of human intervention studies, experience with ethical commission, statistics
- PC skills: Word, Excel, Power Point, SPSS
- Languages: German, English, French
- Experience in planning and execution of meetings and conferences
- Supervision and mentoring of master and bachelor students

Personal interests:

- Hiking, climbing
- Travelling
- Cooking

Publications

Wallner, M., Blassnigg, S. M., Marisch, K., Pappenheim, M. T., Müllner, E., Mölzer, C., Nersesyan, A., Marculescu, R., Doberer, D., Knasmüller, S., Bulmer, A. C. & Wagner, K.-H. 2012. Effects of unconjugated bilirubin on chromosomal damage in individuals with Gilbert`s syndrome measured with the micronucleus cytome assay. *Mutagenesis,* 27, 731-735.

Switzeny, O., Müllner, E., Wagner, K.-H., Brath, H., Aumüller, E. & Haslberger, A. 2012. Vitamin and antioxidant rich diet increases MLH1 promoter DNA methylation in DMT2 subjects. *Clinical Epigenetics*, 4, 19.

Müllner, E., Brath, H., Pleifer, S., Schiermayr, C., Baierl, A., Wallner, M., Fastian, T., Millner, Y., Paller, K., Henriksen, T., Poulsen, H. E., Forster, E. & Wagner, K.-H. 2012. Vegetables and PUFA-rich plant oil reduce DNA strand breaks in individuals with type 2 diabetes. *Molecular Nutrition & Food Research;* 57, 328-338

Müllner, E., Brath, H., Toferer, D., Adrigan, S., Bulla, M.-T., Stieglmayer, R., Wallner, M., Marek, R., Wagner, K.-H. 2013. Genome damage in peripheral blood lymphocytes of diabetic and non-diabetic individuals after intervention with vegetables and plant oil. *Mutagenesis*; 28, 205-211

Congress presentations

Müllner E., Brath H., Wallner M., Wagner K.-H. Vegetables and plant oil and their potential protective effect against DNA damage in individuals with type 2 diabetes. Lifestyle, oxidative stress and diabetes mellitus, November, 8th - 9th, 2012, Modra, Slowakia, oral presentation, abstract: congress Proceedings

Moazzami AA., Müllner E., Brath H., Wagner K-H.: Metabolomics Study of the Effect of Omega-3 and Vegetables on Patients with Type 2 Diabetes Reveals the Metabolic Alteration in Plasma-Phospholipids and Ceramides. 103rd AOCS Annual Meeting, April 29th – May 2nd, 2012, Long Beach, California, USA, oral presentation, Abstract: INFORM

Müllner E., Pleifer S., Schiermayr C., Brath H., Wagner K-H.: The Effect of Vegetables and Plant Oil on DNA Damage in Subjects with Type 2 Diabetes. 103rd AOCS Annual Meeting, April 29th – May 2nd, 2012, Long Beach, California, USA, oral presentation, Abstract: INFORM

Toferer D., Müllner E., Brath H., Forster E., Wagner K.-H. Impact of vegetables and plant oil on the status of vitamin B12, folic acid and homocysteine in type 2 diabetics. ISFE Symposium 2011 – Diet Quality, December, 1st – 2nd, 2011, Vienna, Austria, poster presentation, Abstract: Nutrition

Müllner E., Millner Y., Brath H., Wagner K.-H. Vegetables and plant oil improve vitamin status in subjects with Type 2 Diabetes. ISFE Symposium 2011 – Diet Quality, December, 1st – 2nd, 2011, Vienna, Austria, poster presentation, Abstract: Nutrition

Kienreich N. E., Müllner E., Fastian T., Brath H., Wagner K.-H. An intervention with vegetables and plant oil is able to optimize nutrient intake of type 2 diabetics. ISFE Symposium 2011 – Diet Quality, December, 1st - 2nd, 2011, Vienna, Austria, poster presentation, Abstract: Nutrition

Müllner E., Adrigan S., Bulla M., Pleifer S., Schiermayr C., Stieglmayer R., Brath H., Wagner K.-H. The Impact of a Dietary Intervention on Chromosomal and DNA Damage in Subjects with Type 2 Diabetes. Scientific Symposium Austrian Society of Toxicology (ASTOX) and Emerging Field "Oxidative Stress and DNA Stability" (ANTIOX), November, 24th – 25th, 2011, Vienna, Austria, oral presentation, Abstract: Congress Proceedings

Petschnig A. C., Nitz M., Nersesyan A., Müllner E., Knasmüller S., Brath H., Wagner K.-H. Influence of a dietary intervention on micronuclei frequency in buccal cells of subjects with type 2 patients. Scientific Symposium Austrian Society of Toxicology (ASTOX) and Emerging Field "Oxidative Stress and DNA Stability" (ANTIOX), November, 24th – 25th, 2011, Vienna, Austria, poster presentation, Abstract: Congress Proceedings

Müllner E., Forster E., Brath H., Wagner K.-H. Vegetables an Plant Oil Improve Risk Factor for Secondary Complications in Subjects with Type 2 Diabetes. Austrian Nutrition Society Annual Meeting, Nutrition in Health Promotion and Disease Prevention, October, 19th – 21st, 2011, Vienna, Austria, poster presentation, Abstract: Ernährung/Nutrition

Müllner E., Pleifer S., Schiermayr C., Forster E., Brath H., Wagner K.-H. Vegetables and Plant Oil can protect against DNA damage in Type 2 Diabetes. 9th International Comet Assay Workshop, September, 13th – 16th, 2011, Izmir, Turkey, poster presentation, Abstract: Congress Proceedings

Müllner E., Plasser E., Zajitschek A., Brath H., Wagner K.-H. PUFA rich plant oils and oxidative stress in type 2 diabetics. 7th Euro Fed Lipid Congress, 18th – 21st October, 2009, Graz, Austria; oral presentation, abstract: Book of Abstracts

Wagner K.-H., Plasser E., Müllner E., Forster E. Influence of Plant Oils on Lipid related parameters and Metabolic Control in Type 2 Diabetics. 100th AOCS Annual Meeting, May 3rd - 6th, 2009, Orlando, USA. Abstract: INFORM

Müllner E., Pickova J., Vrede T., Goedkoop W. Fatty Acids in Freshwater Food Webs – Seasonal and among Lake Differences. 6th Euro Fed Lipid Congress, September 7th – 10th 2008, Athens, Greece; poster presentation, abstract: congress proceedings

Plasser E., Müllner E., Brath H., Wagner K.-H. 2009. Impact of Plant Oils on Adiponectin Levels of Type 2 Diabetics. Ann. Nutr. Metabol 55 (suppl 1), 676.

Plasser E., Müllner E., Brath H., Wagner K.-H. 2009. Impact of Plant Oils on the Lipid Profile in Type 2 Diabetics. Ann. Nutr. Metabol 55 (suppl 1), 540.

I

DOI 10.1002/mnfr.201200343 *Mol. Nutr. Food Res.* 2013, *57*, 328–338

RESEARCH ARTICLE

Vegetables and PUFA-rich plant oil reduce DNA strand breaks in individuals with type 2 diabetes

Elisabeth Müllner[1], Helmut Brath[2], Simone Pleifer[1], Christiane Schiermayr[1], Andreas Baierl[1,3], Marlies Wallner[1], Theresia Fastian[1], Yvonne Millner[1], Kristina Paller[1], Trine Henriksen[4], Henrik Enghusen Poulsen[4,5,6], Ernst Forster[2] and Karl-Heinz Wagner[1]

[1] Department of Nutritional Sciences, Emerging Field "Oxidative Stress and DNA Stability" University of Vienna, Austria
[2] Diabetes Outpatient Clinic, Health Centre South, Vienna, Austria
[3] Department of Statistics and Operations Research, University of Vienna, Austria
[4] Laboratory of Clinical Pharmacology Q7642, Rigshospitalet, Copenhagen, Denmark
[5] Department of Clinical Pharmacology, Bispebjerg Hospital, Copenhagen, Denmark
[6] Health Science Faculty, University of Copenhagen, Copenhagen, Denmark

Received: June 1, 2012
Revised: August 23, 2012
Accepted: September 15, 2012

Scope: Type 2 diabetes is a multifactorial disease associated with increased oxidative stress, which may lead to increased DNA damage. The aim of this study was to investigate the effect of a healthy diet on DNA oxidation in diabetics and nondiabetics.

Methods and results: Seventy-six diabetic and 21 nondiabetic individuals participated in this study. All subjects received information about the benefits of a healthy diet, while subjects randomly assigned to the intervention group received additionally 300 g of vegetables and 25 mL PUFA-rich plant oil per day. DNA damage in mononuclear cells (Comet Assay), urinary excretion of 8-oxo-7-hydro-2′-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and glycated hemoglobin (HbA1c) were measured at baseline, after 4, 8 (end of intervention), and 16 weeks. The intervention with vegetables and PUFA-rich oil led to a significant increase in plasma antioxidant concentrations. Diabetic individuals of the intervention group showed a significant reduction in HbA1c and DNA strand breaks. Levels of HbA1c were also improved in diabetics of the information group, but oxidative damage to DNA was not altered. Urinary 8-oxodG and 8-oxoGuo excretion remained unchanged in both groups.

Conclusions: This study provides evidence that a healthy diet rich in antioxidants reduces levels of DNA strand breaks in diabetic individuals.

Keywords:
Diabetes mellitus type 2 / DNA damage / Plant oil / Vegetables

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Type 2 diabetes mellitus (T2DM) is a complex disease characterized by high blood glucose levels resulting from impaired insulin secretion and insulin action, leading to hyperglycemia and a disturbed carbohydrate, fat and protein metabolism [1]. Oxidative stress, mainly caused by hyperglycemia, hyperlipidemia, and hyperinsulinemia is discussed to be involved in the progression of the disease [2] and contributes to the onset of diabetes associated complications [3] and to DNA oxidation [4]. Consistently, the quality of glycemic control plays a major role in preventing DNA oxidation [5–7], implying that an optimum control of factors leading to increased reactive oxygen species production is crucial to diabetes control.

Diet is a cornerstone for the achievement of optimum blood glucose control and lipid concentrations in subjects with T2DM [8]. Based on the nutritional guidelines for the management of diabetes from the American Diabetes Association [9] saturated fatty acid (SFA) intake should be less than

Correspondence: Elisabeth Müllner, Department of Nutritional Sciences, Faculty of Life Sciences, University of Vienna, Althanstraße 14, 1090 Vienna, Austria
E-mail: elisabeth.muellner@univie.ac.at
Fax: +43-1-4277-9549

Abbreviations: **8-oxodG**, 8-oxo-7-hydro-2′-deoxyguanosine; **8-oxoGuo**, 8-oxo-7,8-dihydroguanosine; **FPG**, formamidopyrimidine DNA glycosylase; **HbA1c**, glycated hemoglobin A1c; **ITDM2**, insulin-treated type 2 diabetes; **NIDDM**, non-insulin dependent type 2 diabetes; **PBMCs**, peripheral blood mononuclear cells; **SFAs**, saturated fatty acids; **T2DM**, type 2 diabetes mellitus

7% of the daily energy intake, as this type of fat is linked to unfavorable plasma lipid profile [10]. Scientific evidence even suggests that increased SFA intake is, among other factors, responsible for the increase in diabetes prevalence [11]. Furthermore, an inverse association between the risk of T2DM and the replacement of PUFA for SFA [12], or PUFA intake alone [13] was observed.

Sufficient amounts of vegetables are considered as an essential part of a healthy diet, since they are rich in antioxidants, such as vitamin C, vitamin E, carotenoids, flavonoids, and other polyphenolic compounds. These antioxidants are able to scavenge or inactivate reactive oxygen species and are therefore of special importance to subjects with increased oxidative stress. However, the number of studies reporting positive effects of antioxidant supplementation on oxidative DNA damage is almost equal to the number of studies showing no effects (for review see [14–16]). This raises the question if antioxidant-rich natural foods are more effective than supplements with purified antioxidants.

To the best of our knowledge there are no data available either about the impact of a whole food approach or about the beneficial effects of a healthy diet on oxidative damage to DNA in subjects with T2DM. Therefore a dietary intervention trial with focus on high diet quality, realized through the replacement of SFA by PUFA and the supply of vegetables, was performed. The aims of the study were (i) to assess the potential of the intervention to reduce oxidative damage to DNA under consideration of health status (insulin treated (ITDM2), non-insulin dependent (NIDDM) type 2 diabetes or nondiabetic) and (ii) to investigate the impact of poor or good glycemic control on the response to the intervention. The biomarkers used to study oxidative damage to DNA were 8-oxo-7-hydro-2′-deoxyguanosine (8-oxodG) in urine, and the comet assay in peripheral blood mononuclear cells (PBMC).

2 Materials and methods

2.1 Study population

Seventy-six subjects with established T2DM (according to the definition of the American Diabetes Association [17]) treated with oral glucose-lowering agents (mean age 65.2 ± 7.38 years; 18 males and 22 females) and/or insulin (mean age 65.0 ± 7.68 years; 16 males and 20 females) and 21 nondiabetic subjects (mean age: 62.7 ± 6.30 years, 6 males and 15 females) participated in this study. Subjects were recruited from a local diabetic clinic (Diabetes outpatient Clinic, Health Centre South, Vienna, Austria) during their annual health assessment. The nondiabetic subjects were partners of the diabetic subjects.

All subjects had to have stable metabolic control (constant medication regarding glucose, lipid, and uric acid metabolism), glycated hemoglobin (HbA_{1c}) concentration <9.5%, serum total cholesterol <300 mg/dL (<7.76 mmol/L), serum triglycerides <500 mg/dL (<5.7 mmol/L) and serum creatinine <2.5 mg/dL (<221 μmol/L). Only subjects with stable body weight, constant dietary habits and physical activity levels for at least 4 weeks before entry to the study were included. Subjects who intended to change dietary habits (e.g. intake of additional supplements), body weight or frequency of physical activity within the study period were not allowed to participate. Exclusion criteria also included smoking, intake of fish oil capsules, and other fatty acid supplements. All medical therapies of subjects were continued unchanged throughout the study.

The study protocol was approved by the Ethical Committee of the City of Vienna (EK09–218-VK_NZ). The inclusion criteria were fulfilled by 151 subjects. One hundred twenty gave their written consent. Out of these, 21 withdrew because of health problems unrelated to the study, digestive discomfort, or scheduling conflicts. Two subjects, reported not having diabetes, were excluded because of increased fasting glucose levels. The study was conducted between January and December 2009.

2.2 Dietary intervention

All participants (diabetics and nondiabetics) received information about the beneficial effects of a healthy diet with special focus on the importance of fat quality and the role of vegetables in a balanced diet. Participants were randomly assigned to the "intervention" or "information only" group. Subjects of the "information only" group received only the above mentioned information, while subjects of the "intervention" group received additionally 300 g of vegetables and 25 mL of plant oil (composition see Table 1) per day. The participants were instructed to use the plant oil as replacement for SFA. A reference "cup" and a booklet with recipes, instructions for replacement of SFA and usage of the plant oil

Table 1. Composition of the intervention oil

	Percentage of total fatty acids
C16:0	7.28 ± 0.09
C18:0	2.06 ± 0.07
C18:1n9c	16.4 ± 0.99
C18:1n7c	0.95 ± 0.03
C18:2n6c	61.8 ± 1.0
C18:3n3	11.5 ± 0.43
	Milligram per 100 g
γ-Tocopherol	33.0 ± 1.82
α-Tocopherol	2.67 ± 0.14
Campesterol	6.2 ± 0.45
Campestanol	1.0 ± 0.00
Stigmasterol	1.0 ± 0.00
Sitosterol	116 ± 5.00
Sitostanol	1.2 ± 0.45
Δ 5-avenasterol	11.6 ± 0.89
Cycloartenol + Δ 7-stigmastenol	38 ± 5.70
Minor sterols	7.8 ± 0.84
Total plant sterols	183 ± 2.10

Table 2. Intervention vegetables (amount for 14 days)

Amount (g)	Vegetables
225	Strained spinach
400	Leave spinach
300	Green bean
300	Broccoli
300	Brussels sprouts
300	Soybean
300	Pea
300	Carrot[a)]
400	Romaine lettuce with peas
300	Vegetable mix I (broccoli, cauliflower)
300	Vegetable mix II (broccoli, carrot yellow and orange)
300	Vegetable mix III (carrot, potato, kohlrabi, leek, pea)
300	Vegetable mix VI (pea, carrot, corn)
300	Vegetable mix V (carrot, potato, broccoli, green bean, cauliflower)
800	Roasted vegetables I (carrot, broccoli, cauliflower, pole beans, zucchini)[b)]
800	Roasted vegetables II (carrot, Brussels sprouts, kohlrabi, pole beans)[b)]
400	Ready meal: spinach with potatoes[c)]

a) Was given to subjects additionally, in case of any digestive discomfort.
b) Subjects got roasted vegetables I for the first 14 days and roasted vegetables II for the second 14 days.
c) Contains 230 g of spinach.

(oil was not allowed to be heated up, but added to warm foods) was provided to the participants. A variety of frozen vegetables (Table 2) was given to subjects every 2 weeks. Participants could choose the order of consumption of the provided vegetables. A dietary diary had to be completed, and fatty acid profile, vitamin K, γ-tocopherol, and carotenoid concentrations were measured in plasma to monitor compliance.

The intervention period lasted 8 weeks, followed by a period of 8 weeks in which no intervention foods were provided. Blood samples were taken before the intervention, after 4, 8 (end of intervention period), and 16 weeks.

2.3 Anthropometric measurements

Body height (stadiometer: Seca, Modell 214, Hamburg, Germany) was measured at baseline, and waist circumference and weight (digital scale: Seca, Bella 840, Hamburg, Germany) were measured on every blood sampling day. BMI was calculated as kg/m^2.

2.4 Blood sampling and isolation of PBMCs

Venous blood samples were obtained after an overnight fast using heparin tubes (Becton Dickinson, Schwechat, Austria). Blood was centrifuged at 3000 rpm for 10 min to separate plasma from cells. Fasting plasma glucose, HbA1c and insulin were measured immediately by the laboratory of the Health Centre South, Vienna. In order to determine fatty acid and tocopherol concentrations, plasma was frozen in aliquots at −80°C until analysis.

PBMCs were isolated using Cell Preparation Tubes (Becton Dickinson, Schwechat, Austria). They were separated by centrifugation (3100 rpm, 25 min, room temperature) according to the manufacturer's instructions and washed twice with cold PBS.

2.5 Detection of oxidative damage to DNA in PBMCs

Oxidative damage to DNA and resistance against H_2O_2-induced DNA damage were measured with the single-cell gel electrophoresis assay (Comet Assay) [4]. Briefly, a freshly prepared suspension of PBMCs (approx. 1×10^6 cells/mL in PBS) was mixed with 1% low melting point agarose and put on agarose coated slides (1% normal melting agarose). Four slides were prepared from each cell suspension: "lysis," "buffer," "FPG" (also known as formamidopyrimidine DNA glycosylase), and "H_2O_2". All slides were put in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris base plus 1% Triton X-100 added before use, pH 10) for at least 1 h. Only "H_2O_2" slides were put in 100 μM H_2O_2 solution for 5 min at 4°C before lysis. After lysis, the "buffer" and "FPG" slides were washed three times with enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8) and incubated with 50 μL of enzyme buffer ("buffer" slide) or FPG solution ("FPG" slide) for 30 min at 37°C in a moist box. Slides were put in an electrophoresis tank (CSL-COM40, Biozym, Austria) containing electrophoresis solution (0.3 M NaOH, 1 mM EDTA). After 20 min of unwinding and 30 min of electrophoresis (25 V, 300 mA at 4°C, pH > 13) slides were washed with PBS and dried at room temperature.

Slides were stained with ethidium bromide (20 μg/mL) and the amount of DNA damage was quantified using a fluorescence microscope coupled with an imaging analysis system (Komet 5.5, Kinetinc Imaging, Liverpool, UK). For each sample, two replicate gels were analyzed and the mean %DNA in tail of 50 comets per gel was calculated. Slides were blinded before microscopic analysis. The net amount of FPG-sensitive sites was determined by calculating the difference between the obtained DNA damage after FPG and buffer treatment.

2.6 Detection of oxidative damage to DNA and RNA in urine

Urine samples were collected on the day of blood samplings, aliquoted and stored at –20°C until analysis. 8-oxodG and 8-oxo-7,8-dihydroguanosine (8-oxoGuo) were measured at the Laboratory of Clinical Pharmacology, Rigshospitalet, in Copenhagen, using a validated method for ultraperformance LC and MS/MS [18]. 8-oxodG and 8-oxoGuo were normalized against urinary creatinine concentration determined by the Jaffe reaction.

2.7 Biochemical analyses

HbA1c was analyzed in whole blood by HPLC (Automated Glycohemoglobin Analyzer HLC-723G8, Tosoh, Tokyo, Japan). Fasting plasma glucose was determined enzymatically by the hexokinase method (Aeroset, Abbott Diagnostics, IL, USA) and plasma insulin concentrations were measured on an Immulite 2000 immunochemistry system using reagents and calibrator obtained from the instrument supplier (Siemens Medical Solutions Diagnostics, Flanders, USA). The principle of the method is a solid-phase, tow-site chemiluminescent immunometric assay. Homeostasis model assessment of insulin resistance was calculated as the product of fasting plasma glucose (mmol/L) and insulin (μU/mL) concentrations, divided by 22.5 as originally described by Matthews et al. [19].

2.8 Measurement of compliance markers

The fatty acid profile in plasma was determined by a GC equipped with a flame ionization detector [20]. Identification of fatty acids was based on the comparison of the samples' retention times to those of a 37 Component FAME Mix standard (Supelco, Bellefonte, USA). TotalChrom Workstation 6.3.0, PE Nelson, Perkin Elmer was used for peak integration.

Plasma concentrations of vitamin K, α- and γ-tocopherol, retinol, lutein, β-cryptoxanthin, lycopene, α-, and β-carotene were determined by RP-HPLC [21]. Each vitamin was quantitated on the basis of peak area using a calibration curve generated from standard solutions. To ensure quality control a control plasma sample was run throughout the study. CVs for all fatty acids and vitamins were <10%.

2.9 Oil analyses

The fatty acid pattern and tocopherol concentrations of the plant oil were determined with the same method as described above. Sterol content of the plant oil was analyzed by capillary GC at the Department of Food and Environmental Sciences, Helsinki, Finland [22]. The concentration of the given sterols or stanols is the sum of their free and esterified forms. The samples were measured in triplicate and the sterol values obtained were accepted if RSD <5%.

2.10 Statistical analyses

Statistical analyses were performed using SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). Normal distribution within the data set was tested by the Kolmogorov–Smirnov test. Independent samples *t*-test (for parametric data) or Mann–Whitney U-test (for nonparametric data) was conducted to assess differences between two groups. Multiple group comparisons were performed with one-way ANOVA followed by Bonferroni post hoc test. If the assumptions of normality and homogeneity of variance were violated, Kruskal–Wallis *H*-test was used followed by Dunnett-T3 as post hoc test.

The effect of the intervention was assessed by (i) comparing the changes after 4 and 8 weeks between the two treatment (information or intervention) groups (univariate ANOVA with treatment as fixed factor) and by (ii) comparing baseline values with values after 4 and 8 weeks of intervention (univariate ANOVA). Time point of blood sampling and squared time point of blood sampling were used as covariates. The effect of the intervention was assessed in the total study group and in the subgroups with different health status (ITDM2, NIDDM, nondiabetic). Data of the subgroups are shown, if significant changes were found. Statistical tests were carried out on log-transformed data (e.g. log strand breaks week 8 – log strand breaks week 0). Furthermore, the impact of the intervention was analyzed in participants with poor and good glycemic control (HbA1c below or above 7%) based on the above-mentioned procedure. Pearson correlation and Spearman rank correlation were used to evaluate the association between variables. Results were considered significant at $P < 0.05$.

3 Results

3.1 Baseline characteristics of the study population

In total 97 subjects completed the study. There were no significant differences between subjects of the intervention and information group at baseline (Table 3), apart from γ-tocopherol, which was significantly higher ($P = 0.026$), and vitamin K, which trended to be higher ($P = 0.059$) in subjects of the intervention group.

Baseline concentrations of fasting plasma glucose, HbA1c and BMI were significantly lower in nondiabetic subjects compared to both ITDM2 and NIDDM subjects (Supporting Information Table 1). Diabetes duration and HbA1c levels differed significantly between ITDM2 and NIDDM subjects. Significant differences in levels of 8-oxoGuo were observed between nondiabetic and NIDDM subjects. Furthermore, subjects with HbA1c concentrations even at 6.5% had significantly higher global RNA oxidation compared to subjects with HbA1c <6.5% (8-oxoGuo: 3.69 ± 1.51 vs. 3.04 ± 0.85 nmol/mmol creatinine, respectively; $P = 0.011$). Further analyses about the impact of glycemic control on oxidative damage to DNA revealed higher levels of FPG-sensitive sites in subjects in the highest tertile of fasting plasma glucose (>8.6 mmol/L), compared to the lowest tertile (<6.39 mmol/L; FPG-sensitive sites in the highest and lowest tertile: 2.85 ± 1.99 vs. 4.47 ± 2.95% DNA in tail; $P = 0.031$).

Table 3. Baseline characteristics of participants in the information and intervention group

	Intervention	Information	*P*-value[a)]
Number (ITDM2/NIDDM/nondiabetic)	66 (25/29/12)	31 (11/11/9)	
BMI (kg/m^2)	32.5 ± 5.79	32.2 ± 7.03	0.934
Fasting plasma glucose (mmol/L)	8.13 ± 2.37	7.41 ± 2.07	0.261
HbA1c (%)	7.26 ± 1.08	7.01 ± 1.05	0.711
Strand breaks (% DNA in tail)	5.74 ± 2.32	4.63 ± 0.98	0.595
Resistance to H_2O_2 (% DNA in tail)	23.6 ± 10.7	21.2 ± 7.08	0.400
FPG-sensitive sites (% DNA in tail)	3.62 ± 2.31	3.84 ± 2.93	0.202
8-oxoGuo (nmol/mmol creatinine)	3.66 ± 1.44	3.17 ± 1.17	0.615
8-oxodG (nmol/mmol creatinine)	2.18 ± 0.79	1.87 ± 0.78	0.838
Lutein (μmol/L)	0.22 ± 0.17	0.19 ± 0.09	0.292
α-Carotene (μmol/L)	0.09 ± 0.04	0.09 ± 0.04	0.898
b-Carotene (μmol/L)	0.25 ± 0.25	0.25 ± 0.20	0.746
γ-Tocopherol (μmol/L)	1.60 ± 0.68	2.04 ± 1.09	0.026
Vitamin K (nmol/L)	0.74 ± 0.59	1.05 ± 0.88	0.059

ITDM2, insulin-treated type 2 diabetes mellitus; NIDDM, noninsulin dependent type 2 diabetes mellitus; HbA1c, glycated haemoglobin. Data are presented as means ± SD.
a) *P*-values for difference between subjects of information and intervention group adjusted for time point and squared time point of blood sampling.

3.2 Dietary compliance

To asses dietary compliance the fatty acid profile and levels of γ-tocopherol (for the oil intake) and carotenoids and vitamin K (for the vegetable intake) were measured. In subjects of the intervention group plasma levels of linoleic and linolenic acid, γ-tocopherol, lutein, α-carotene, β-carotene, and vitamin K were significantly increased after 4 and 8 weeks of intervention compared with baseline (Table 4). Analysis of the groups with different health status confirmed the

Table 4. Changes in compliance parameters after 4 and 8 weeks in the intervention and information group

	Baseline	Change after 4 weeks	Change after 8 weeks	*P* after 4 weeks[a)]	*P* after 8 weeks[b)]
Lutein (μmol/l)					
Information	0.19±0.09	+0±0.06	+0±0.06	0.509	0.466
Intervention	0.22±0.17	+0.14±0.12[c)]	+0.13±0.12[d)]	<0.001	<0.001
α-Carotene (μmol/l)					
Information	0.09±0.04	+0.01±0.03	+0.01±0.06	0.484	0.165
Intervention	0.09±0.04	+0.07±0.05[c)]	+0.08±0.06[d)]	<0.001	<0.001
β-Carotene (μmol/l)					
Information	0.25±0.2	+0.03±0.11	+0.07±0.17	0.273	0.007
Intervention	0.25±0.25	+0.12±0.12[c)]	+0.13±0.16[d)]	<0.001	<0.001
γ-Tocopherol (μmol/l)					
Information	2.04±1.09	+0.12±0.8	−0.03±0.89	0.186	0.929
Intervention	1.6±0.68	+0.69±0.56[c)]	+0.59±0.68[d)]	<0.001	<0.001
Vitamin K (nmol/l)					
Information	1.05±0.88	−0.31±0.78	−0.16±0.75	0.206	0.559
Intervention	0.74±0.59	+1.08±1.56[c)]	+1.13±1.58[d)]	<0.001	<0.001
Linoleic acid (%)[e)]					
Information	24.86±3.77	−0.54±1.91	−0.14±2.39	0.169	0.795
Intervention	24.85±4.1	+3.72±3.23[c)]	+3.51±3.09[d)]	<0.001	<0.001
α-Linolenic acid (%)[e)]					
Information	0.51±0.19	+0±0.22	−0.01±0.15	0.902	0.939
Intervention	0.5±0.18	+0.3±0.26[c)]	+0.31±0.3[d)]	<0.001	<0.001

Data are presented as means ± SD.
a) *P*-values for differences between baseline and week 4.
b) *P*-values for differences between baseline and week 8.
c) Significant difference between subjects of information and intervention group after 4 weeks.
d) Significant difference between subjects of information and intervention group after 8 weeks.
e) Reported as percentage of total fatty acids.
P-values were calculated with univariate ANOVA after adjustment for time point and squared time point of blood sampling.

significant increase in all vegetable (Supporting Information Fig. 1) and oil compliance parameters (Supporting Information Fig. 2) after 4 and 8 weeks in ITDM2, NIDDM, and nondiabetic subjects. Interestingly, a significantly weaker increase in plasma α- and β-carotene was observed after 8 weeks of intervention in diabetic individuals (α-carotene: ITDM2: +0.04 ± 0.06 μmol/L; NIDDM: +0.06 ± 0.07 μmol/L; β-carotene: ITDM2: +0.05 ± 0.13 μmol/L; NIDDM: +0.1 ± 0.12 μmol/L) compared to nondiabetic subjects (α-carotene: +0.09 ± 0.09 μmol/L, β-carotene: +0.24 ± 0.21 μmol/L).

None of the compounds changed significantly in the information group, apart from β-carotene, which showed a slight but significant increase after 8 weeks. Analysis of the groups with different health status revealed significant changes in β-carotene only in healthy individuals (Supporting Information Fig. 1). The changes in all the compliance markers were significantly higher in the intervention group, after 4 and 8 weeks, compared with the information group (Table 4).

Since subjects were instructed to consume their habitual diet after the intervention period, most of the compliance markers returned to their baseline levels at week 16. Only linolenic acid, α-, and β-carotene remained significantly higher than at baseline. However, there were no significant differences in compliance markers between intervention and information group at week 16 (data not shown).

3.3 Glycemic control

Significant reductions in HbA1c levels were observed after 4 and 8 weeks of oil and vegetable consumption in ITDM2 and NIDDM subjects. HbA1c levels remained constant in nondiabetic individuals. In the information group, HbA1c levels were not significantly changed after 4 weeks, but significantly improved in ITDM2 and NIDDM subjects after 8 weeks (Supporting Information Fig. 3A–C). Consequently, changes in HbA1c levels were not significantly different between the two treatment groups (Table 5).

After return to the usual diet (week 16), subjects of the intervention group still had lower HbA1c levels than at baseline (week 0: 7.3 ± 1.1%, week 16: 7.1 ± 1.1%; $P = 0.065$). The reduction was significant in ITDM2 subjects (week 0: 8.1 ± 0.9%, week 16: 7.9 ± 0.9%; $P = 0.031$) but not in NIDDM and nondiabetic individuals (data not shown).

3.4 Levels of oxidative damage to DNA in PBMCs (Comet Assay)

Strand breaks, H_2O_2 sensitivity, and FPG-sensitive sites, were measured at baseline, and after 4 and 8 weeks of intervention (Table 5). Daily vegetable and oil consumption reduced DNA strand breaks by 13.8% after 4 ($P < 0.001$) and by 17.1% after 8 weeks ($P < 0.001$) of intervention compared to baseline values. In the subgroups of the intervention group with different health status, the reduction of DNA strand breaks was significant in ITDM2 and NIDDM subjects, but not in nondiabetic individuals. No effects on levels of DNA strand breaks were observed in the information group (Fig. 1, Supporting Information Fig. 3D–F).

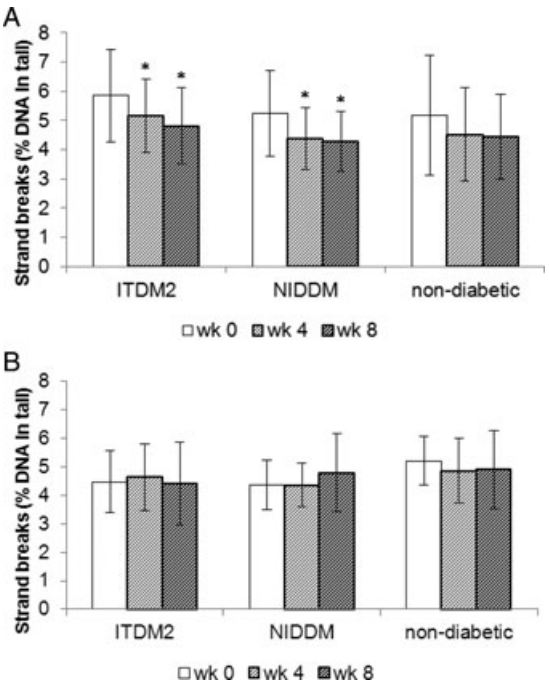


Figure 1. Changes in DNA strand breaks assessed by comet assay in (**A**) subjects of the intervention and (**B**) information group with different health status (insulin treated (ITDM2), non-insulin dependent (NIDDM) subjects with type 2 diabetes and nondiabetic individuals) from baseline (wk 0) to week 4 (wk 4) and week 8 (wk 8). Values are means ± SD. *Significantly different from baseline value (wk 0) of the corresponding group.

FPG-sensitive sites were significantly reduced (−13.2%, $P = 0.037$) in the intervention group after 4 weeks of intervention, whereas after 8 weeks, levels were not significantly different from baseline (−3.31%; Table 5). The reduction of FPG-sensitive sites after 4 weeks was in fact only significant in NIDDM subjects (week 0: 3.46 ± 2.56% DNA in tail, week 4: 2.47 ± 1.68% DNA in tail; $P = 0.048$).

The reductions in DNA strand breaks, FPG-, and H_2O_2-sensitive sites after 4 and 8 weeks were not significantly different between information and intervention group.

After return to the usual diet, levels of FPG- and H_2O_2-sensitive sites were not significantly different from baseline (data not shown). Levels of strand breaks increased at week 16, but were still significantly lower than at baseline (week 0: 5.4 ± 1.6% DNA in tail; week 16: 4.7 ± 1.3% DNA in tail).

3.5 Urinary excretion of 8-oxodG and 8-oxoGuo

Levels of 8-oxodG and 8-oxoGuo, markers for DNA and RNA oxidation, respectively, were not affected by the dietary

Table 5. Changes in HbA1c, levels of DNA strand breaks, FPG-sensitive sites, H_2O_2-sensitivity and urinary excretion of 8-oxodG and 8-oxoGuo after 4 and 8 weeks in the intervention and information group

	Baseline	Change after 4 weeks	Change after 8 weeks	*P* after 4 weeks[a)]	*P* after 8 weeks[b)]
HbA1c (%)					
Information	7.01±1.05	−0.07±0.22	−0.16±0.25	0.118	0.002
Intervention	7.26±1.08	−0.17±0.34	−0.19±0.47	<0.001	0.002
Strand breaks (% DNA in tail)					
Information	4.63±0.98	−0.03±1.16	+0.07±1.61	0.785	0.953
Intervention	5.45±2.32	−0.73±1.5	−0.93±1.74	<0.001	<0.001
FPG-sensitive sites (% DNA in tail)					
Information	3.84±2.93	−0.26±2.91	−0.39±3.83	0.364	0.794
Intervention	3.62±2.31	−0.54±2.63	−0.15±3.56	0.037	0.558
H_2O_2-sensitive sites (% DNA in tail)					
Information	21.15±7.08	−0.28±5.6	+0.7±4.29	0.804	0.244
Intervention	23.55±10.66	−0.2±7.46	−1.56±7.65	0.225	0.795
8-oxodG (nmol/mmol creatinine)					
Information	1.87±0.78	+0.02±0.31	+0.02±0.39	0.297	0.528
Intervention	2.18±0.79	−0.01±0.53	−0.05±0.66	0.432	0.482
8-oxoGuo (nmol/mmol creatinine)					
Information	3.17±1.17	−0.01±0.86	+0.02±1.34	0.529	0.659
Intervention	3.66±1.44	+0.18±0.99	+0.2±1.43	0.293	0.155

Data are presented as means ± SD.
a) *P*-value for difference between baseline and week 4.
b) *P*-value for differences between baseline and week 8.
P-values were calculated with univariate ANOVA after adjustment for time point and squared time point of blood sampling.

intervention. There was no difference in 8-oxodG and 8-oxoGuo excretion after 4, 8, and 16 weeks between the two treatment groups.

3.6 Response to the dietary intervention based on preintervention glycemic control

To test the hypothesis whether the response to the dietary intervention is related to the quality of glycemic control, subjects were divided into groups with HbA1c below or above 7%, which is the HbA1c target level recommended by the American Diabetes Association [23].

In the intervention group 22 of 25 ITDM2, 12 of 29 NIDDM and none of the 12 nondiabetic subjects had HbA1c levels >7%. The effect of the dietary intervention on DNA strand breaks was significant after 4 and 8 weeks of intervention in both subgroups (HbA1c below or above 7%). The effect was stronger in subjects with HbA1c > 7%; however the changes were not significantly different from subjects with HbA1c levels <7% (Table 6). Changes in H_2O_2 sensitivity after 4 and 8 weeks of intervention were significantly different between the HbA1c subgroups due to the reduction in H_2O_2 sensitivity in subjects with HbA1c >7% and the slight increase in subjects with HbA1c ≤7%. FPG-sensitive sites were significantly reduced after 4 but not 8 weeks of intervention in subjects with HbA1c >7%. Changes in FPG-sensitive sites and urinary excretion of 8-oxodG and 8-oxoGuo were not significantly different between subjects above or below the recommended HbA1c level.

Analysis of the groups with different health status revealed significant reductions of DNA strand breaks in NIDDM (in both, HbA1c above ($n = 12$) and below 7% ($n = 17$)) and ITDM2 subjects (HbA1c above 7% ($n = 22$); Supporting Information Fig. 4). Furthermore, significant reductions in FPG-sensitive sites in ITDM2 subjects with HbA1c >7% after 8 weeks (week 0: 4.42 ± 2.22% DNA in tail, week 8: 3.17 ± 2.62% DNA in tail) and in 8-oxodG levels in NIDDM subjects with HbA1c ≤7% (week 0: 2.48 ± 0.93 nmol/mmol creatinine, week 4: 2.16 ± 0.98 nmol/mmol creatinine, week 8: 2.05 ± 0.89 nmol/mmol creatinine; $P = 0.015$, $P = 0.010$, respectively) were observed.

4 Discussion

T2DM is a rapidly increasing epidemic affecting people worldwide [24]. Dietary habits are among other factors intensively discussed to be responsible for the onset and the progression of the disease. A disturbed redox status, which is linked to T2DM itself and diet quality, leads to oxidative stress and can trigger oxidative DNA damage. Therefore, the aim

Table 6. Changes in levels of DNA strand breaks, FPG-sensitive sites, H_2O_2-sensitivity and urinary excretion of 8-oxodG and 8-oxoGuo after 4 and 8 weeks of intervention with vegetables and plant oil in subjects with poor ($n = 34$) and good glycemic control ($n = 32$)

	Baseline	Change after 4 weeks	Change after 8 weeks	*P* after 4 weeks[a)]	*P* after 8 weeks[b)]
Strand breaks (% DNA in tail)					
HbA1c ≤7%	5.27±1.66	−0.59±1.35	−0.86±1.43	0.019	0.002
HbA1c >7%	6.18±2.76	−1.47±2.92	−1.57±3.03	<0.001	<0.001
FPG-sensitive sites (% DNA in tail)					
HbA1c ≤7%	3.49±2.42	−0.43±2.97	+0.28±3.74	0.453	0.906
HbA1c >7%	3.75±2.23	−0.65±2.34	−0.63±3.36	0.037	0.373
H_2O_2-sensitive sites (% DNA in tail)					
HbA1c ≤7%	21.94±10.41	+1.76±6.36	+0.56±7.57	0.035	0.256
HbA1c >7%	25.07±10.83	−2.04±8.03[c)]	−3.56±7.27[d)]	0.526	0.024
8-oxodG (nmol/mmol creatinine)					
HbA1c ≤7%	2.3±0.84	−0.13±0.46	−0.13±0.7	0.118	0.200
HbA1c >7%	2.05±0.73	+0.11±0.57	+0.02±0.61	0.839	0.771
8-oxoGuo (nmol/mmol creatinine)					
HbA1c ≤7%	3.44±1.02	+0.18±0.99	+0.27±0.97	0.333	0.195
HbA1c >7%	3.86±1.75	+0.17±1.02	+0.13±1.81	0.633	0.408

Data are presented as means ± SD.
a) *P*-values for differences between baseline and 4 week.
b) *P*-value for difference between baseline and 8 week.
c) Significantly different from subjects with HbA1c ≤7% after 4 weeks.
d) Significantly different from subjects with HbA1c ≤7% after 8 weeks.
P-values were calculated with univariate ANOVA after adjustment for time point and squared time point of blood sampling.

of the present intervention study was to investigate whether a healthy diet, with focus on vegetable supply and fat quality (replacing SFA by PUFA), is able to reduce oxidative damage to DNA in subjects with T2DM. Furthermore baseline values of oxidative damage to DNA were compared between nondiabetic individuals and subjects with T2DM.

In the present study, DNA damage in lymphocytes did not differ between diabetic and nondiabetic subjects. Studies, which have investigated this issue, have reported conflicting results [25–29], which can be explained by the complexity of diabetes, the big interindividual variations, leading to incomparability of study groups.

Also body weight, especially obesity, is discussed as a confounding factor regarding oxidative damage to DNA [30]. However within this trial no associations between BMI and oxidative damage to DNA were observed, either in diabetic or in nondiabetic subjects. A positive association between BMI and DNA strand breaks was described in individuals with type I diabetes [31] and in a study population consisting of healthy subjects, patients with impaired glucose regulation, and newly diagnosed diabetes [29]. On the contrary, no association between DNA strand breaks [32], or levels of 8-oxodG and BMI were reported in healthy individuals [33] and within a case–control study consisting of healthy individuals and breast cancer patients [34].

The difference in FPG-sensitive sites between ITDM2, NIDDM, and healthy subjects did not reach statistical significance ($P = 0.068$). An association between fasting plasma glucose and oxidized purines, already described in other studies [5, 27, 28, 31], could be confirmed within this trial by the increase in FPG-sensitive sites with increasing fasting plasma glucose tertile. This result supports the suggestion that FPG-sensitive sites specifically reflect damage resulting from hyperglycemia [31].

8-oxoGuo, a biomarker for global RNA oxidation, was recently suggested as a clinical biomarker for the assessment of mortality risk in newly diagnosed T2DM patients [35]. Within our trial, global RNA oxidation levels were significantly lower in nondiabetic subjects compared to ITDM2 and NIDDM subjects. Furthermore, glycemic control can be suggested as one determinant of global RNA oxidation, since subjects with HbA1c concentrations even at 6.5% had significantly higher 8-oxoGuo levels compared to subjects with HbA1c <6.5%.

A very novel finding of the present study was the reduction in strand breaks, measured by Comet Assay, in T2DM subjects after the intervention with vegetables and plant oil, while levels were not significantly changed in nondiabetic individuals. To the best of our knowledge, no data about the impact of vegetables on DNA damage in T2DM subjects are published. Supplementation trials with vitamin E [36] or flavonols [37] are consistent with our findings and showed a reduction in strand breaks in T2DM subjects. Intervention studies in healthy individuals with a vegetable/fruit concentrate [38]; 600 g of fruit and vegetables [39]; 200 g of

cooked minced carrots [40]; a tomato-based drink [41]; 2, 5, or 8 servings/day of vegetables and fruit [42]; 300 g of Brussels sprouts [43]; or 225 g of spinach [44] however reported no effect on DNA strand breaks. The discrepancy in effects between diabetic and nondiabetic individuals in the present study might be due to significantly lower baseline levels of α- and β-carotene in ITDM2 and NIDDM subjects compared to nondiabetics, indicating that subjects with low antioxidant status benefit more from the intervention. We also observed a significantly weaker increase in plasma α- and β-carotene after 8 weeks of intervention in diabetic individuals compared to nondiabetic subjects, which might reflect the use of the known antioxidants as radical scavengers. Similar observations were made in intervention studies with watercress [45] or with 300–400 g of vegetables and fruit [46] in smokers, who showed a significantly lower plasma response in antioxidants after the intervention compared to nonsmokers.

Moller et al. also suggested that the oxidative burden at baseline has a major impact on the effect of dietary interventions [47]. Within this trial subjects of the information group had significantly higher plasma γ-tocopherol levels at baseline compared to the intervention group. Furthermore, levels of DNA strand breaks were lower, although not significantly, in the information group compared to the intervention group. One cannot rule out the possibility that these factors influence the outcome of the dietary intervention.

Oxidative burden is increased in T2DM patients, especially in those with poor glycemic control via autoxidation of glucose, the activation of protein kinase C, the increased production of advanced glycation end products and the increase in polyol and hexosamine pathway activity [3, 48]. Within this trial the impact of glycemic control on the outcome of the intervention with vegetables and plant oil could be confirmed via the significant reduction in FPG- and H_2O_2-sensitive sites in subjects (ITDM2 and NIDDM) with HbA1c >7% after 4 and 8 weeks, respectively. The reduction in FPG-sensitive sites was also significant after 8 weeks in ITDM2 but not in IDDM patients with HbA1c >7%. The mechanisms, responsible for the reduction in oxidative damage to DNA after vegetable and oil consumption are so far unknown. The reduction may be related to the improvement in antioxidant status [49] and the decreased HbA1c concentration, indicating improved glycemic control. However, our data further suggest that an improvement in glycemic control alone, as seen in the information group, is not sufficient to reduce oxidative damage to DNA.

Another possible mechanism against oxidative damage to DNA is cellular repair [50]. 8-oxodG and 8-oxoGuo are excreted into the urine as a result of DNA repair [18, 51], and are important biomarkers of oxidative DNA and RNA damage [18]. However, 8-oxodG and 8-oxoGuo remained constant during the intervention. Similar findings were reported by Freese et al. [52], who found no effect of a diet rich in fruit and vegetables (total amount 1059 g/10 MJ) combined with PUFA (11% of energy) on 8-oxodG levels in healthy subjects.

At week 16 (return to usual diet), levels of strand breaks increased compared to week 8 (end of intervention) but were still significantly lower compared to baseline. After week 8, intervention foods were no longer provided and oil bottles were recollected. Interestingly, HbA1c and dietary antioxidants were still improved at week 16 compared to baseline, suggesting that subjects of the intervention group kept their healthier diet, at least in part. This was also confirmed by food frequency questionnaires, which were collected at baseline and week 16, showing a significantly higher consumption of vegetables at week 16 compared to baseline (data not shown).

In summary, a healthy diet, rich in vegetables and with a considerable amount of PUFA, replacing SFA for 8 weeks, decreases DNA strand breaks. This positive effect might be related to the reduction in HbA1c and the improvement in antioxidant status, strengthening the idea that diet quality is important in treatment of T2DM. However, the present results also suggest that providing information to subjects with T2DM about the beneficial effects of a healthy diet once, particularly pointing out the role of PUFA and differently colored vegetables, improves glycemic control, but does not alter antioxidant status or oxidative damage to DNA.

This work was funded by EU, through the cross-border cooperation program Slovakia–Austria 2007–2013 (http://www.sk-at.eu) *and the Austrian Ministry of Health. The authors thank Prof. Dr. Vieno Piironen and Dr. Anna-Maija Lampi for conducting the sterol analysis of the plant oil and IGLO-Austria for providing the vegetables. We gratefully acknowledge the support of Prof. Andrew Collins who kindly provided FPG enzyme.*

The authors have declared no conflict of interest.

5 References

[1] American Diabetes Association, Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2012, *35*, S64–S71.

[2] Folli, F., Corradi, D., Fanti, P., Davalli, A. et al., The role of oxidative stress in the pathogenesis of type 2 diabetes mellitus micro-and macrovascular complications: avenues for a mechanistic-based therapeutic approach. *Curr. Diabetes Rev.* 2011, *7*, 313–324.

[3] Giacco, F., Brownlee, M., Oxidative stress and diabetic complications. *Circ. Res.* 2010, *107*, 1058–1070.

[4] Azqueta, A., Shaposhnikov, S., Collins, A. R., DNA oxidation: investigating its key role in environmental mutagenesis with the comet assay. *Mut. Res.* 2009, *674*, 101–108.

[5] Dinçer, Y., Akçay, T., Alademir, Z., Ilkova, H., Assessment of DNA base oxidation and glutathione level in patients with type 2 diabetes. *Mut. Res.* 2002, *505*, 75–81.

[6] Broedbaek, K., Henriksen, T., Weimann, A., Petersen, M. et al., Long-term effects of irbesartan treatment and smoking on nucleic acid oxidation in patients with type 2 diabetes and microalbuminuria: an irbesartan in patients with type 2 diabetes and microalbuminuria (IRMA 2) substudy. *Diabetes Care* 2011, *34*, 1192–1198.

[7] Nishikawa, T., Sasahara, T., Kiritoshi, S., Sonoda, K. et al., Evaluation of urinary 8-hydroxydeoxy-guanosine as a novel biomarker of macrovascular complications in type 2 diabetes. *Diabetes Care* 2003, *26*, 1507–1512.

[8] Nolan, C. J., Damm, P., Prentki, M., Type 2 diabetes across generations: from pathophysiology to prevention and management. *Lancet* 2011, *378*, 169–181.

[9] Bantle, J. P., Wylie-Rosett, J., Albright, A. L., Apovian, C. M. et al., Nutrition recommendations and interventions for diabetes: a position statement of the American Diabetes Association. *Diabetes Care* 2008, *31*, S61–S78.

[10] Mensink, R. P., Zock, P. L., Kester, A. D. M., Katan, M. B., Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *Am. J. Clin. Nutr.* 2003, *77*, 1146–1155.

[11] Kolb, H., Mandrup-Poulsen, T., The global diabetes epidemic as a consequence of lifestyle-induced low-grade inflammation. *Diabetologia* 2010, *53*, 10–20.

[12] Meyer, K. A., Kushi, L. H., Jacobs, D. R. Jr., Folsom, A. R., Dietary fat and incidence of type 2 diabetes in older Iowa women. *Diabetes Care* 2001, *24*, 1528–1535.

[13] Salmerón, J., Hu, F. B., Manson, J. E., Stampfer, M. J. et al., Dietary fat intake and risk of type 2 diabetes in women. *Am. J. Clin. Nutr.* 2001, *73*, 1019–1026.

[14] Hoelzl, C., Knasmüller, S., Mišík, M., Collins, A. et al., Use of single cell gel electrophoresis assays for the detection of DNA-protective effects of dietary factors in humans: recent results and trends. *Mut. Res.* 2009, *681*, 68–79.

[15] Loft, S., Møller, P., Cooke, M. S., Rozalski, R. et al., Antioxidant vitamins and cancer risk: is oxidative damage to DNA a relevant biomarker? *Eur. J. Nutr.* 2008, *47*, 19–28.

[16] Wasson, G. R., McKelvey-Martin, V. J., Downes, C. S., The use of the comet assay in the study of human nutrition and cancer. *Mutagenesis* 2008, *23*, 153–162.

[17] American Diabetes Association, Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2009, *32*, S62–S67.

[18] Henriksen, T., Hillestrøm, P. R., Poulsen, H. E., Weimann, A., Automated method for the direct analysis of 8-oxoguanosine and 8-oxo-2′-deoxyguanosine in human urine using ultraperformance liquid chromatography and tandem mass spectrometry. *Free Radic. Biol. Med.* 2009, *47*, 629–635.

[19] Matthews, D. R., Hosker, J. P., Rudenski, A. S., Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985, *28*, 412–419.

[20] Wagner, K. H., Auer, E., Elmadfa, I., Content of trans fatty acids in margarines, plant oils, fried products and chocolate spreads in Austria. *Eur. Food Res. Technol.* 2000, *210*, 237–241.

[21] Jakob, E., Elmadfa, I., Rapid HPLC assay for the assessment of vitamin K1, A, E and beta-carotene status in children (7–19 years). *Int J Vit Nutr Res* 1995, *65*, 31–35.

[22] Piironen, V., Toivo, J., Lampi, A. M., New data for cholesterol contents in meat, fish, milk, eggs and their products consumed in Finland. *J. Food Compost. Anal.* 2002, *15*, 705–713.

[23] American Diabetes Association, Standards of medical care in diabetes – 2012. *Diabetes Care* 2012, *35*, S11–S63.

[24] Wagner, K.-H., Brath, H., A global view on the development of non communicable diseases. *Prev. Med.* 2012, *54* (Supplement), S38–S41.

[25] Arif, M., Islam, M. R., Waise, T. M. Z., Hassan, F. et al., DNA damage and plasma antioxidant indices in Bangladeshi type 2 diabetic patients. *Diabetes Metab.* 2010, *36*, 51–57.

[26] Ibarra-Costilla, E., Cerda-Flores, R. M., Dávila-Rodríguez, M. I., Samayo-Reyes, A. et al., DNA damage evaluated by comet assay in Mexican patients with type 2 diabetes mellitus. *Acta Diabetol.* 2010, *47*, S111–S116.

[27] Lodovici, M., Giovannelli, L., Pitozzi, V., Bigagli, E. et al., Oxidative DNA damage and plasma antioxidant capacity in type 2 diabetic patients with good and poor glycaemic control. *Mut. Res.* 2008, *638*, 98–102.

[28] Pitozzi, V., Giovannelli, L., Bardini, G., Rotella, C. M. et al., Oxidative DNA damage in peripheral blood cells in type 2 diabetes mellitus: higher vulnerability of polymorphonuclear leukocytes. *Mut. Res.* 2003, *529*, 129–133.

[29] Song, F., Jia, W., Yao, Y., Hu, Y. et al., Oxidative stress, antioxidant status and DNA damage in patients with impaired glucose regulation and newly diagnosed type 2 diabetes. *Clin. Sci.* 2007, *112*, 599–606.

[30] Demirbag, R., Yilmaz, R., Kocyigit, A., Relationship between DNA damage, total antioxidant capacity and coronary artery disease. *Mut. Res.* 2005, *570*, 197–203.

[31] Collins, A. R., Rašlová, K., Somorovská, M., Petrovská, H. et al., DNA damage in diabetes: correlation with a clinical marker. *Free Radic. Biol. Med.* 1998, *25*, 373–377.

[32] Giovannelli, L., Saieva, C., Masala, G., Testa, G. et al., Nutritional and lifestyle determinants of DNA oxidative damage: a study in a Mediterranean population. *Carcinogenesis* 2002, *23*, 1483–1489.

[33] Pilger, A., Germadnik, D., Riedel, K., Meger-Kossien, I. et al., Longitudinal study of urinary 8-hydroxy-2′-deoxyguanosine excretion in healthy adults. *Free Radic. Res.* 2001, *35*, 273–280.

[34] Rossner, P. Jr., Gammon, M. D., Terry, M. B., Agrawal, M. et al., Relationship between urinary 15-F2t-isoprostane and 8-oxodeoxyguanosine levels and breast cancer risk. *Cancer Epidemiol. Biomarkers Prev.* 2006, *15*, 639–644.

[35] Broedbaek, K., Siersma, V., Henriksen, T., Weimann, A. et al., Urinary markers of nucleic acid oxidation and long-term mortality of newly diagnosed type 2 diabetic patients. *Diabetes Care* 2011, *34*, 2594–2596.

[36] Şardaş, S., Yilmaz, M., Öztok, U., Çakir, N. et al., Assessment of DNA strand breakage by comet assay in diabetic patients and the role of antioxidant supplementation. *Mut. Res.* 2001, *490*, 123–129.

[37] Lean, M. E. J., Noroozi, M., Kelly, I., Burns, J. et al., Dietary flavonols protect diabetic human lymphocytes against oxidative damage to DNA. *Diabetes* 1999, *48*, 176–181.

[38] Van Den Berg, R., Van Vliet, T., Broekmans, W. M. R., Cnubben, N. H. P. et al., A vegetable/fruit concentrate with high antioxidant capacity has no effect on biomarkers of

antioxidant status in male smokers. *J. Nutr.* 2001, *131*, 1714–1722.

[39] Møller, P., Vogel, U., Pedersen, A., Dragsted, L. O. et al., No effect of 600 grams fruit and vegetables per day on oxidative DNA damage and repair in healthy nonsmokers. *Cancer Epidemiol. Biomarkers Prev.* 2003, *12*, 1016–1022.

[40] Astley, S. B., Elliott, R. M., Archer, D. B., Southon, S., Evidence that dietary supplementation with carotenoids and carotenoid-rich foods modulates the DNA damage: repair balance in human lymphocytes. *Br. J. Nutr.* 2004, *91*, 63–72.

[41] Riso, P., Visioli, F., Grande, S., Guarnieri, S. et al., Effect of a tomato-based drink on markers of inflammation, immunomodulation, and oxidative stress. *J. Agric. Food Chem.* 2006, *54*, 2563–2566.

[42] Briviba, K., Bub, A., Möseneder, J., Schwerdtle, T. et al., No differences in DNA damage and antioxidant capacity between intervention groups of healthy, nonsmoking men receiving 2, 5, or 8 servings/day of vegetables and fruit. *Nutr. Cancer* 2008, *60*, 164–170.

[43] Hoelzl, C., Glatt, H., Meinl, W., Sontag, G. et al., Consumption of Brussels sprouts protects peripheral human lymphocytes against 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhiP) and oxidative DNA-damage: results of a controlled human intervention trial. *Mol. Nutr. Food Res.* 2008, *52*, 330–341.

[44] Moser, B., Szekeres, T., Bieglmayer, C., Wagner, K. H. et al., Impact of spinach consumption on DNA stability in peripheral lymphocytes and on biochemical blood parameters: results of a human intervention trial. *Eur. J. Nutr.* 2011, *50*, 587–594.

[45] Gill, C. I. R., Haldar, S., Boyd, L. A., Bennett, R. et al., Watercress supplementation in diet reduces lymphocyte DNA damage and alters blood antioxidant status in healthy adults. *Am. J. Clin. Nutr.* 2007, *85*, 504–510.

[46] Chopra, M., O'Neill, M. E., Keogh, N., Wortley, G. et al., Influence of increased fruit and vegetable intake on plasma and lipoprotein carotenoids and LDL oxidation in smokers and nonsmokers. *Clin. Chem.* 2000, *46*, 1818–1829.

[47] Møller, P., Loft, S., Interventions with antioxidants and nutrients in relation to oxidative DNA damage and repair. *Mut. Res.* 2004, *551*, 79–89.

[48] Brownlee, M., Biochemistry and molecular cell biology of diabetic complications. *Nature* 2001, *414*, 813–820.

[49] Torbergsen, A. C., Collins, A. R., Recovery of human lymphocytes from oxidative DNA damage; the apparent enhancement of DNA repair by carotenoids is probably simply an antioxidant effect. *Eur. J. Nutr.* 2000, *39*, 80–85.

[50] Collins, A. R., Harrington, V., Drew, J., Melvin, R., Nutritional modulation of DNA repair in a human intervention study. *Carcinogenesis* 2003, *24*, 511–515.

[51] Cooke, M. S., Evans, M. D., Dove, R., Rozalski, R. et al., DNA repair is responsible for the presence of oxidatively damaged DNA lesions in urine. *Mut. Res.* 2005, *574*, 58–66.

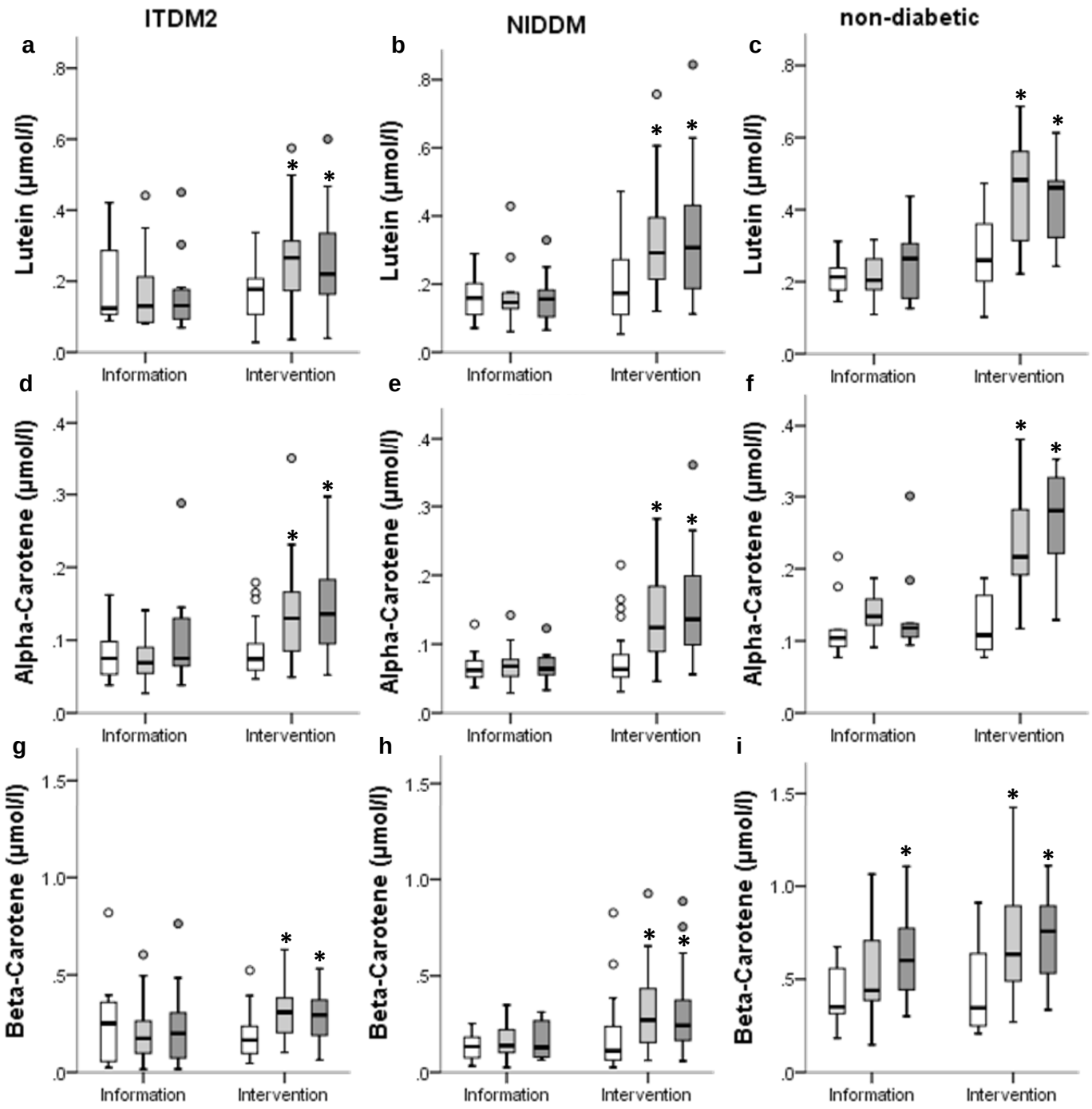
[52] Freese, R., Dragsted, L. O., Loft, S., Mutanen, M., No effect on oxidative stress biomarkers by modified intakes of polyunsaturated fatty acids or vegetables and fruit. *Eur. J. Clin. Nutr.* 2008, *62*, 1151–1153.

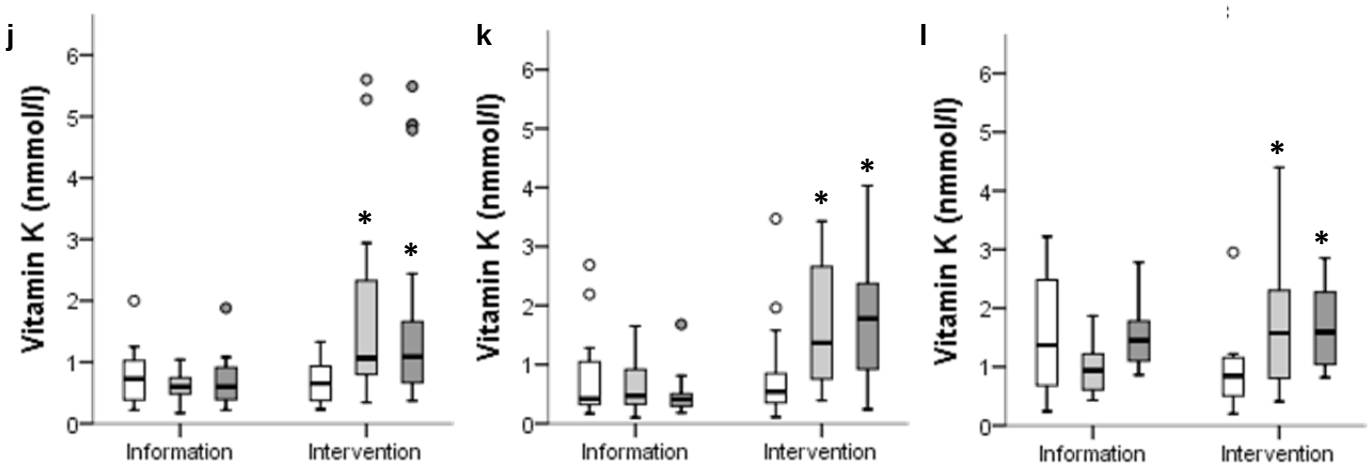
Supporting Information Table 1. Baseline characteristics of the study population

	ITDM2	NIDDM	Non-diabetic	P^1
Number (male/female)	36 (16/20)	40 (18/22)	21 (6/15)	
Age (years)	65±7.68	65.2±7.38	62.7±6.3	0.406
BMI (kg/m^2)	33.8±6.46[a]	33.3±6.15[a]	28.4±3.84[b]	0.002
Fasting plasma glucose (mmol/l)	9.07±2.54[a]	8.04±1.73[a]	5.63±0.5[b]	<0.001
HbA1c (%)	8.02±0.84[a]	7.12±0.7[b]	5.79±0.26[b]	<0.001
Diabetes duration (years)	18.4±10.1[a]	10.8±8[b]		<0.001
Strand breaks (% DNA in tail)	5.94±2.73	5±1.38	5.19±1.66	0.190
H_2O_2 sensitivity (% DNA in tail)	22.8±9.27	22.9±10.1	22.7±10.2	0.998
FPG-sensitive sites (% DNA in tail)	4.37±2.88	3.52±2.44	2.81±1.47	0.068
8-oxodG (nmol/mmol creatinine)	1.89±0.79	2.31±0.78	2±0.75	0.073
8-oxo-Guo (nmol/mmol creatinine)	3.69±1.81[a]	3.66±1.15[a]	2.93±0.62[b]	0.046

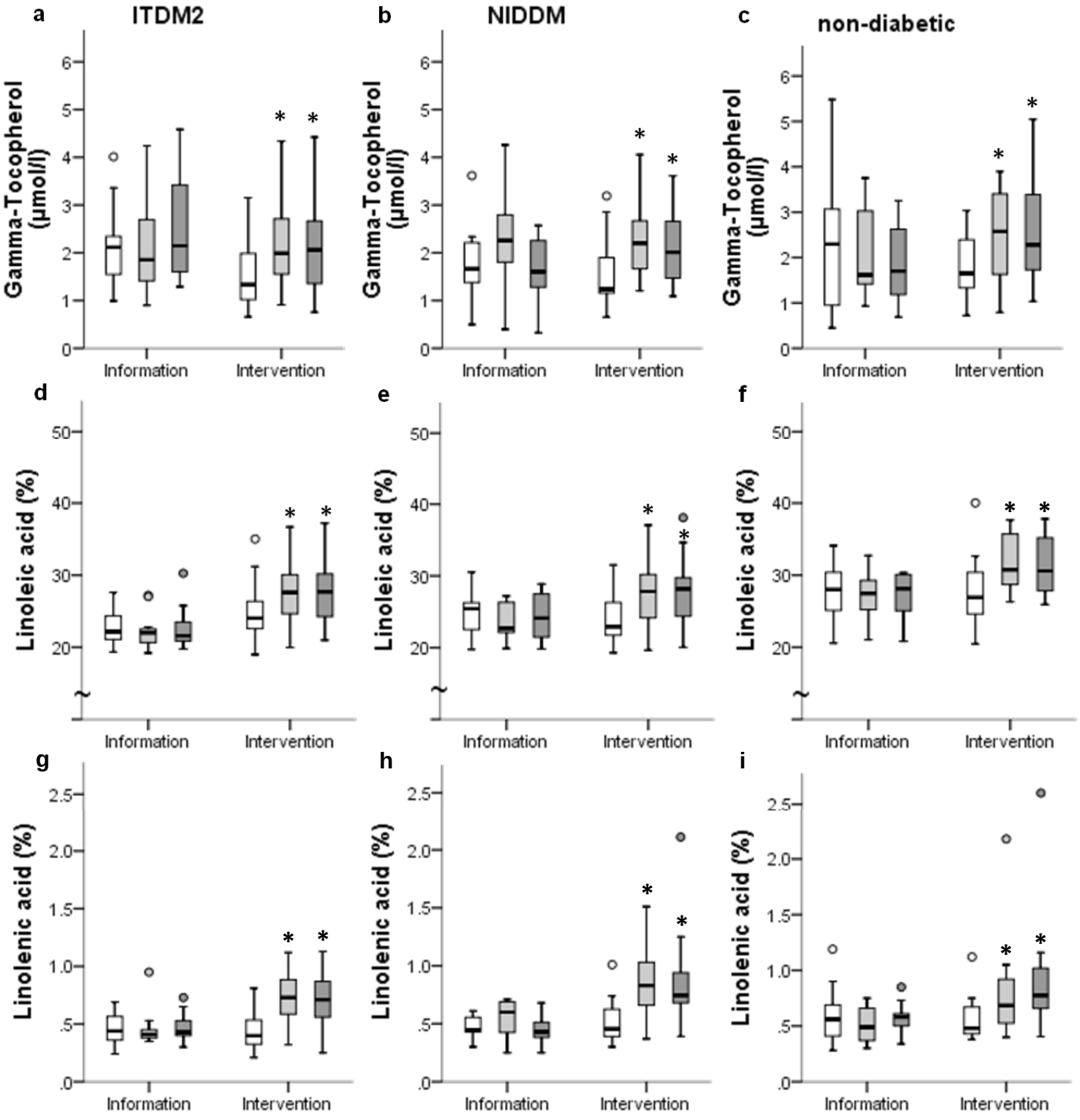
ITDM2, insulin-treated type 2 diabetes mellitus; NIDDM, non-insulin dependent type 2 diabetes mellitus; BMI, body mass index, HbA1c, glycated haemoglobin. Data are presented as means ± SD

[1] ANOVA with Bonferroni as post hoc test (age, BMI, strand breaks, H_2O_2 sensitivity, 8-oxodG) or Kruskal-Wallis-Test with Dunnett-T3 as post hoc test (fasting plasma glucose, HbA1c, 8-oxo-Guo) or unpaired t-test (diabetes duration). Parameters within a row with different superscript letter are significantly different.

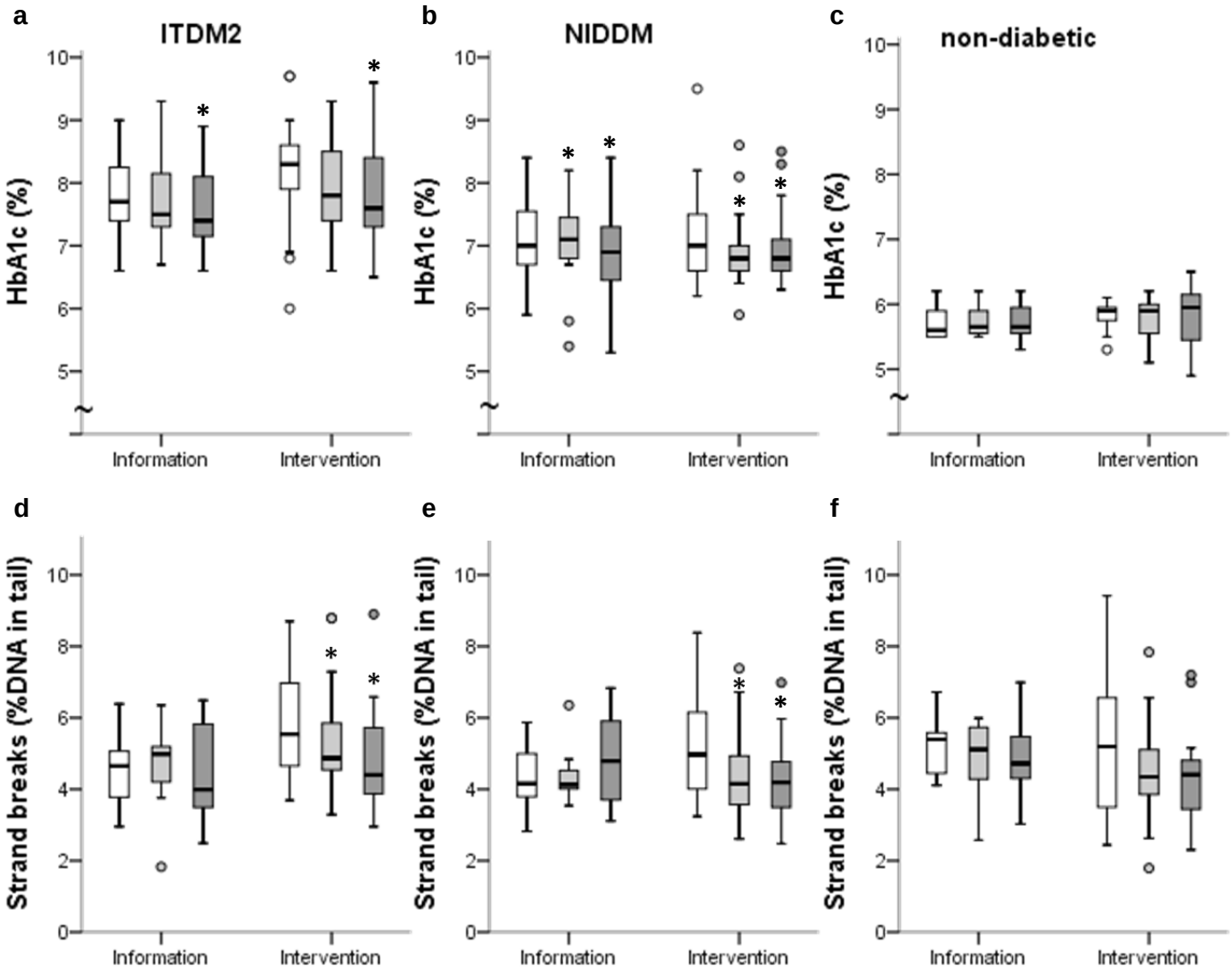

ITDM2
NIDDM
non-diabetic
a
b
c
d
e
f
g
h
i
Lutein (µmol/l)
Alpha-Carotene (µmol/l)
Beta-Carotene (µmol/l)
Information
Intervention



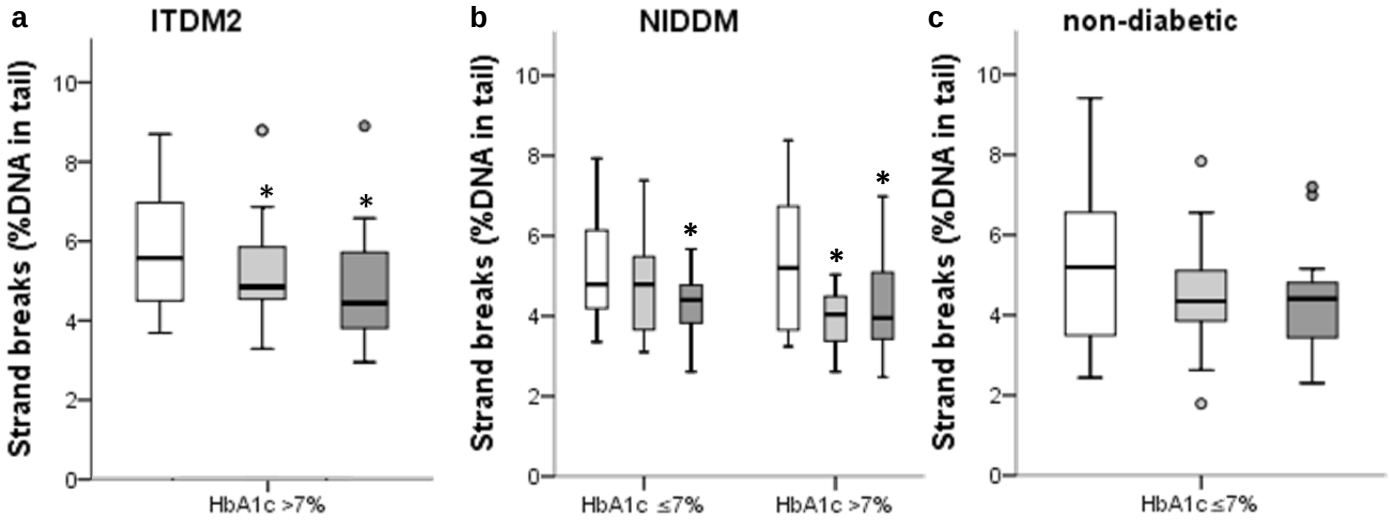
Supporting Information Figure 1. Box plots of vegetable compliance parameters at baseline (white) after 4 (light grey) and 8 weeks (dark grey) in individuals with insulin treated type 2 diabetes (ITDM2 – left column: figure a, d, g, j), non-insulin dependent type 2 diabetes (NIDDM – middle column: figure b, e, h, k) and non-diabetic subjects (right column: figure c, f, i, l) of the information and intervention group. *Significantly different from baseline value of the corresponding group (univariate ANOVA after adjustment for time point and squared time point of blood sampling).



Supporting Information Figure 2. Box plots of oil compliance parameters at baseline (white) after 4 (light grey) and 8 weeks (dark grey) in individuals with insulin treated type 2 diabetes (ITDM2 – left column: figure a, d, g), non-insulin dependent type 2 diabetes (NIDDM – middle column: figure b, e, h) and non-diabetic subjects (right column: figure c, f, i) of the information and intervention group. *Significantly different from baseline value of the corresponding group (univariate ANOVA after adjustment for time point and squared time point of blood sampling).



Supporting Information Figure 3. Box plots of (a – c) HbA1c and (d – f) DNA strand breaks at baseline (white) after 4 (light grey) and 8 weeks (dark grey) in individuals with insulin treated type 2 diabetes (ITDM2 – left column: figure a, d, g), non-insulin dependent type 2 diabetes (NIDDM – middle column: figure b, e, h) and non-diabetic subjects (right column: figure c, f, i) of the information and intervention group. *Significantly different from baseline value of the corresponding group (univariate ANOVA after adjustment for time point and squared time point of blood sampling).



Supporting Information Figure 4. Box plots of DNA strand breaks at baseline (white) after 4 (light grey) and 8 weeks (dark grey) in individuals with insulin treated type 2 diabetes (ITDM2: figure a), non-insulin dependent type 2 diabetes (NIDDM: figure b) and non-diabetic subjects (figure c) with HbA1c above or below 7%. Only 3 ITDM2 had HbA1c levels ≤7% and none of the non-diabetic subjects had HbA1c levels >7% and therefore no data for these subgroups are shown. *Significantly different from baseline value of the corresponding group (univariate ANOVA after adjustment for time point and squared time point of blood sampling).

II

Mutagenesis vol. 28 no. 2 pp. 205–211, 2013
Advance Access publication 21 January 2013
doi:10.1093/mutage/ges073

Genome damage in peripheral blood lymphocytes of diabetic and non-diabetic individuals after intervention with vegetables and plant oil

Elisabeth Müllner[1,*], Helmut Brath[2], Daniela Toferer[1], Sonja Adrigan[1], Marie-Therese Bulla[1], Roswitha Stieglmayer[1], Marlies Wallner[1], Reinhard Marek[2] and Karl-Heinz Wagner[1]

[1]Department of Nutritional Sciences, Emerging Field "Oxidative Stress and DNA Stability", University of Vienna, Austria and [2]Diabetes Outpatient Clinic, Health Centre South, Vienna, Austria

*To whom correspondence should be addressed. Department of Nutritional Sciences, University of Vienna, Althanstraße 14, 1090 Vienna, Austria. Tel: +43 1 4277 54933; Fax: +43 1 4277 9549; Email: elisabeth.muellner@univie.ac.at

Received on October 10, 2012; revised on November 7, 2012; accepted on November 9, 2012

Recent studies suggest increased cancer risk in patients with type 2 diabetes mellitus (T2DM) compared with healthy individuals. The present study aims to assess whether T2DM is associated with increased genome instability and whether a healthy diet with natural foods can improve genome stability in peripheral blood lymphocytes (PBLs). Seventy-six diabetic and 21 non-diabetic individuals were randomly assigned to either an 'intervention' or an 'information only' group. All participants received information about the beneficial effects of a healthy diet, while subjects of the intervention group received additionally 300 g of vegetables and 25 ml of plant oil rich in polyunsaturated fatty acids per day for 8 weeks. Chromosomal damage was assessed using the cytokinesis-block micronucleus (MN) cytome assay. Levels of chromosomal damage did not differ between diabetic and non-diabetic individuals. However, diabetic individuals with MN frequency above the high 50th percentile had significantly higher levels of fasting plasma glucose, glycosylated haemoglobin and were at higher risk for cardiovascular disease (CVD), assessed by the Framingham general cardiovascular risk score. Non-diabetic individuals with MN frequency above the 50th percentile had significantly lower vitamin B12 levels. The intervention with vegetables and plant oil led to significant increases in folate, γ-tocopherol, α- and β-carotene while vitamin B12 was significantly reduced. Levels of chromosomal damage were not altered, only apoptosis was slightly increased. The results suggest interactions between glycaemic control, CVD risk and genome stability in individuals with T2DM. However, a healthy diet does not improve genome damage in PBLs.

Introduction

Type 2 diabetes mellitus (T2DM) is an increasing health problem worldwide and is associated with severe complications (1). Hyperglycaemia and insulin resistance are the main characteristics of the disease and, based on epidemiological evidence, result in increased risk for cardiovascular disease (CVD) (2,3) and cancer (4).

Recent meta-analysis investigating the association between T2DM and non-Hodgkin lymphoma, leukaemia, myeloma (5), hepatocellular carcinoma (6), pancreatic cancer (7) and colorectal cancer (8,9) reported increased cancer risk in individuals with T2DM. However, results of epidemiological studies regarding cancer risk in T2DM patients are not consistent (10) and it is still unclear whether the association between diabetes and cancer is direct (due to hyperglycaemia) or indirect (due to shared risk factors such as obesity or smoking) (11,12).

A frequently used method to assess genome stability is the cytokinesis-block micronucleus cytome (CBMN) assay. The use of micronuclei (MN) frequency as a biomarker for cancer risk is based on theoretical evidence confirming the causal role of MN induction in cancer development (13) and epidemiological evidence reporting increased incidence of all cancers for subjects with high MN frequency (14).

Shettigar *et al*. (15) investigated MN frequency in T2DM and healthy individuals and reported no differences between the two groups; however, MN frequency was associated with elevated glycosylated haemoglobin (HbA_{1c}) (15). Within another case control study, higher MN frequency in T2DM patients with no micro- or macrovascular complications compared with healthy individuals was found (16). In patients with coronary artery disease, MN frequency was significantly higher in the presence of T2DM (17). CVD is suggested to be a major determinant of MN frequency, based on correlations between MN frequency and both the prevalence (18,19) and the severity of coronary artery disease (18).

Growing evidence suggests that MN frequency can be modulated by dietary factors (20–23). Micronutrients play an essential role in the protection against genome damage since they are required as cofactors in DNA synthesis and repair (21). A population study with healthy individuals suggests that at least nine micronutrients (calcium, folate, nicotinic acid, vitamin E, retinol, β-carotene, panthothenic acid, biotin and riboflavin) affect genome stability (24). Furthermore, the beneficial effect of multiple antioxidants on MN frequency was confirmed in human intervention studies (24–26).

Within this study, we investigated the effect of a healthy diet, realised through the supply of mainly green vegetables and the replacement of saturated fatty acids (SFA) by polyunsaturated fatty acids (PUFA) on chromosomal damage in T2DM and non-diabetic participants. The study aimed (i) to investigate differences between diabetic and non-diabetic individuals at baseline under consideration of CVD risk (27) and (ii) to assess the impact of a dietary intervention on chromosomal damage. To the best of our knowledge, this is the first dietary intervention study in T2DM patients with natural foods investigating the complete pattern of markers that are considered as relevant for carcinogenesis [MN, nuclear buds (NBuds), nucleoplasmic bridges (NPBs), cellular apoptosis and necrosis].

Materials and methods

Participants

Patients with T2DM (36 treated with insulin, 40 treated with oral antidiabetic medication) were recruited from a local diabetes clinic (Diabetes Outpatient

Clinic, Health Centre South, Vienna, Austria). Non-diabetic individuals ($n = 21$) were partners of the diabetic subjects.

Individuals with T2DM had to have stable metabolic control (constant medication regarding glucose, lipid and uric acid metabolism), HbA_{1c} concentration <9.5%, serum total cholesterol (TC) <300 mg/dl (<7.76 mmol/l), serum triglycerides <500 mg/dl (<5.7 mmol/l) and serum creatinine <2.5 mg/dl (<221 µmol/l). Non-diabetic individuals had to be free of diabetes [based on definitions of the American Diabetes Association (ADA)] (28) and were not allowed to take any glucose lowering drugs. Otherwise the same exclusion criteria were applied.

All subjects had to have stable body weight, constant dietary habits and physical activity levels for at least 4 weeks before entry to the study. Subjects who intended to change dietary habits, frequency of physical activity or body weight within the study period were not allowed to participate. Further exclusion criteria were smoking, intake of fish oil capsules and other fatty acids. All medical therapies of subjects were continued unchanged throughout the study.

The study protocol was approved by the Ethical Committee of the City of Vienna (EK09-218-VK_NZ). The inclusion criteria were fulfilled by 151 subjects, of whom 120 gave their written consent. Out of these, 21 withdrew because of health problems unrelated to the study, digestive discomfort or scheduling conflicts. Two participants of the non-diabetic group were excluded because of elevated fasting glucose levels. The trial is registered on Current Controlled Trials (ISRCTN53451803).

Study design and dietary intervention

All participants (diabetics and non-diabetics) received information about the beneficial effects of a healthy diet with special focus on the importance of fat quality and the role of vegetables in a balanced diet. Participants were randomly assigned to the 'intervention' or 'information only' group. Subjects of the 'information only' group received only the above-mentioned information, while subjects of the 'intervention' group received additionally 300 g of vegetables and 25 ml of PUFA-rich walnut oil per day [details on sterol content and fatty acid profile see (29)]. Participants were instructed to use the plant oil as replacement for SFA. A reference 'cup' and a booklet with recipes, instructions for replacement of SFA and usage of the plant oil (oil was not allowed to be heated up, but added to warm foods) was provided to the participants. A variety of frozen vegetables was given to the subjects every 2 weeks (list of supplied vegetables and calculated dietary composition are shown in the online supplement; supplementary Table I, available at *Mutagenesis* online). A dietary diary had to be completed, and fatty acid profile and various vitamin concentrations were measured to monitor compliance.

The intervention period lasted 8 weeks, followed by a period of 8 weeks in which no intervention foods were provided. Blood samples were taken before the intervention, after 4, 8 (end of intervention period) and 16 weeks. The CBMN assay was performed at baseline and after 8 weeks and therefore only data of these two time points are presented. Furthermore, participants had to complete a blood glucose profile, a dietary diary at all four blood sampling time points and a food frequency questionnaire (FFQ) at baseline and week 16.

Anthropometry, blood pressure and Framingham general cardiovascular risk

Waist circumference, body height (stadiometer: Seca, Modell 214, Hamburg, Germany) and body weight (digital scale: Seca, Bella 840, Hamburg, Germany) were measured. Body mass index (BMI) was calculated as kilogram per square metre. Furthermore, three blood pressure measurements (BpTRU Medical Devices, Coquitlam, BC, Canada) with 2-min intervals in between (the mean of the last two measurements was used) were obtained after at least 5-min rest with the subject in a seated position.

The Framingham general cardiovascular risk (27) was estimated using following variables: age, sex, tobacco use, treated/untreated systolic blood pressure, diabetes and lipid profile [TC, high-density lipoprotein (HDL) cholesterol].

Blood sampling and isolation of PBMCs

Venous blood samples were obtained after an overnight fast using heparin or serum tubes (Becton Dickinson, Schwechat, Austria). After centrifugation, serum and plasma were aliquoted, used fresh or frozen at −80°C for further analysis. Erythrocytes were washed three times with isotonic phosphate buffer, aliquoted and stored at −80°C.

Biochemical analyses

Fasting plasma glucose, HbA_{1c}, TC and HDL cholesterol were measured immediately by the laboratory of the Health Centre South, Vienna. HbA_{1c} was analysed in whole blood by high-performance liquid chromatography (HPLC) (automated Glycohemoglobin Analyzer HLC-723G8; Tosoh, Tokyo, Japan). Fasting plasma glucose was determined enzymatically by the hexokinase method (Aeroset, Abbott Diagnostics, Abbott Park, IL, USA). Serum TC and HDL cholesterol levels were measured enzymatically by an automated method (Aeroset, Abbott Laboratories, North Chicago, IL, USA) using commercial kits (Abbott).

Measurement of fatty acids, vitamins, antioxidants and homocysteine

The fatty acid profile in plasma was determined by a gas chromatograph equipped with a flame ionisation detector (30). Identification of fatty acids was based on the samples' retention times compared with a 37 Component FAME Mix standard (Supelco, Bellefonte, USA). TotalChrom Workstation 6.3.0, Perkin Elmer was used for peak integration.

Plasma concentrations of vitamin K, α- and γ-tocopherol, retinol, lutein, α- and β-carotene were determined by reverse-phase HPLC (31). Each vitamin was quantitated on the basis of peak area using a calibration curve generated from standard solutions.

Plasma concentrations of vitamin B12 and folic acid were measured according to routine diagnostic tests on Siemens Immulite 2000 analyzer (Siemens Healthcare Diagnostics, Tarrytown, USA) at the laboratory of the Health Centre South, Vienna using chemiluminescent enzyme immunoassay. Folic acid in erythrocytes was measured with radioimmunoassay. Standard curves were drawn and sample values calculated according to the protocol published by the kit producer (MP Biomedicals, Germany). Plasma homocysteine was measured by reversed-phase HPLC with fluorescence detector (emission wavelength: 515 nm; excitation wavelength: 385 nm) on a LiChrosphere column (5 µm, 125 × 4 mm; Merck, Hitachi, LaChrom, Austria). Potassium hydrogen phosphate buffer with 4% acetonitrile was used as mobile phase (32).

CBMN assay

PBMCs were isolated using Cell Preparation Tubes (Becton Dickinson, Schwechat, Austria) following the manufacturer's instructions. The CBMN assay was performed according to the protocol of Fenech (33). A concentration of 1×10^6 cells/ml in culture medium was stimulated to mitotic division with phytohaemagglutinin (PHA; PAA, Austria) and incubated at 37°C, 5% CO_2. Forty-four hours after PHA stimulation, cytocalasin B (Sigma Aldrich, Austria) was added to block cytokinesis. After a total of 72 h of incubation, cells were transferred to slides using cytocentrifuge (Shandon Cytospin 3) and stained (Diff-Qick; Medion Diagnostics, Switzerland). For each subject, duplicates were performed and two slides of each duplicate were prepared. The frequency of binucleated cells (BN) with MN, NPBs and NBuds was scored in 2000 BN according to published criteria (33,34). Furthermore, the number of necrotic and apoptotic cells as well as the nuclear division index (NDI) (33) were determined.

To minimise the potential confounding effect of sex, MN frequency was adjusted for sex by using the ratio of mean MN frequency in males and females as correction factor as suggested by Fenech *et al*. (35). Age was not included, since no correlation between MN frequency and age was observed, due to the narrow age range of the participants.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). Normal distribution within the data set was tested by histograms and the Kolmogorov–Smirnov test. Independent samples *t*-test (for parametric data) or Mann–Whitney *U*-test (for non-parametric data) was conducted to assess differences between two groups. Multiple group comparisons were performed with one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test or Kruskal–Wallis *H*-test if the assumptions of normality or homogeneity of variance were not met.

The effect of the intervention was assessed by (i) comparing baseline values with values after 8 weeks of intervention (univariate ANOVA) and (ii) by comparing the change after 8 weeks of intervention between the two treatment groups (univariate ANOVA, with treatment and health status as fixed factors). Statistical tests were carried out on log-transformed data (e.g. log MN week 8 – log MN week 0). Pearson correlation and Spearman rank correlation were used to evaluate the association between variables. Results were considered significant at $P < 0.05$. The statistical power of the study was based on a minimal detectable change of 5.8 BN with MN per 1000 BN from baseline to week 8, a standard deviation of 8.1 and a significance level of 5%. A reduction by 5.8 BN with MN per 1000 BN was considered to be pathologically significant, since such a change can be induced by exposure to 10 cGy of ionizing radiation (36). The sum of diabetic and non-diabetic participants was divided into intervention and information group, and thereby a statistical power of >90% for each subgroup was reached.

Results

Baseline data

In total, 97 individuals completed the study. There were no significant differences in clinical features, nutritional status or chromosomal damage between the intervention and information group at baseline. Only age and NDI were slightly, but significantly different between the two groups (Table I).

As expected, fasting plasma glucose, HbA_{1c} and CVD risk were significantly higher in diabetic compared with non-diabetic individuals (8.52 ± 2.20 vs. 5.63 ± 0.50 mmol/l, $P < 0.001$; 7.55 ± 0.89 vs. 5.79 ± 0.26%, $P < 0.001$; 21.67 ± 8.01 vs. 11.61 ± 6.59, $P < 0.001$, respectively). Furthermore, vitamin B12 was significantly lower in diabetic participants (309 ± 139 vs. 405 ± 152 pmol/l; $P = 0.011$). The percentage number of participants in the two treatment groups with low levels of vitamin B12, plasma and red blood cell folate are shown in Table II.

Correlation analysis identified relationships between fasting plasma glucose and MN frequency ($r = 0.283$, $P = 0.006$, adjusted for sex). Also self-measured fasting glucose levels were correlated to MN frequency ($r = 0.359$, $P = 0.007$, adjusted for sex). Regarding age, plasma and erythrocyte nutrient status, there were no correlations with any CBMN assay parameter.

Comparisons of baseline data between male and female volunteers showed a 44% greater MN frequency in females relative to males (25.0 ± 8.4 vs. 17.4 ± 5.1; $P < 0.001$), a 21% greater number of necrotic cells (1.54 ± 0.79 vs. 1.21 ± 0.69%; $P = 0.037$) and 12% higher homocysteine levels (13.4 ± 2.74 vs. 11.8 ± 2.93 µmol/l; $P = 0.010$) in males compared with females.

To assess the influence of CVD risk, glucose and nutrient status on MN frequency, participants were stratified on the basis of MN frequency at baseline using the 50th percentile. The results (Figure 1) showed that diabetic subjects with high MN frequency have significantly higher fasting plasma glucose ($P = 0.004$), HbA_{1c} ($P = 0.048$) and CVD risk ($P = 0.021$). Non-diabetic participants with high MN frequency had significantly lower plasma vitamin B12 levels ($P = 0.005$) compared with non-diabetic individuals with low MN frequency.

Table I. Characteristics of participants at baseline

	Information	Intervention	*P*
Number[a] (ITDM2/NIDDM/non-diabetic)	31 (11/11/9)	66 (25/29/12)	
Age (years)	61.7 ± 6.66	65.9 ± 7.19	0.006
BMI (kg/m^2)	32.2 ± 7.03	32.5 ± 5.79	0.820
Fasting plasma glucose (mmol/l)	7.41 ± 2.07	8.13 ± 2.37	0.150
HbA_{1c} (%)	7.01 ± 1.05	7.26 ± 1.08	0.295
Vitamin B12 (pmol/l)	338 ± 150	325 ± 146	0.689
Plasma folate (nmol/l)	18.1 ± 8.37	20.8 ± 8.58	0.157
Red blood cell folate (nmol/l)	318 ± 175	355 ± 232	0.269
Homocysteine (µmol/l)	13.1 ± 3.27	12.2 ± 2.75	0.162
BN with MN per 1000 BN	22.9 ± 7.6	21.2 ± 8.28	0.350
BN with NPBs per 1000 BN	1.78 ± 1.31	1.65 ± 1.27	0.617
BN with NBuds per 1000 BN	4.44 ± 2.89	4.4 ± 2.98	0.950
Apoptosis (%)	1.36 ± 0.63	1.25 ± 0.5	0.386
Necrosis (%)	1.19 ± 0.7	1.44 ± 0.76	0.145
NDI	1.98 ± 0.13	1.92 ± 0.14	0.042

Data are presented as means ± SD. ITDM2, insulin-treated type 2 diabetes mellitus; NIDDM, non-insulin-dependent type 2 diabetes mellitus; BMI, body mass index; BN, binucleated cells; HbA_{1c}, glycosylated haemoglobin; MN, micronuclei; NBud, nuclear bud; NDI, nuclear division index; NPB, nucleoplasmic bridge.
[a]In the article, ITDM2 and NIDDM are referred to as 'diabetics' (information group: 22 diabetic subjects, intervention group: 54 diabetic subjects).

Table II. Percentage of participants with low folate and vitamin B12 levels of the information and intervention group at baseline and after 8 weeks

	Information		Intervention	
	Baseline	Week 8	Baseline	Week 8
Red blood cell folate (<317 nmol/l)	21 (14/7)[a]	27 (19/8)	36 (31/5)	26 (24/2)
Plasma folate (6.8–13.4 nmol/l)[b]	12 (9/3)	11 (10/1)	12 (12/0)	6 (6/0)
Vitamin B12 (<150 pmol/l)	1 (1/0)	1 (1/0)	3 (3/0)	8 (7/1)

[a]Numbers are given in percentage of total participants; the percentages of diabetic and non-diabetic individuals are indicated in brackets.
[b]None of the participants was plasma folate deficient (<6.8 nmol/l).

Intervention study

The measurements of fatty acid profile, tocopherols, carotenoids, folic acid and vitamin K confirmed good dietary compliance: plasma levels of linoleic (+13%; $P < 0.001$) and linolenic acid (+60%; $P < 0.001$), γ-tocopherol (+37%; $P < 0.001$), lutein (+58%; $P < 0.001$), α-carotene (+92%; $P < 0.001$), β-carotene (+53%; $P < 0.001$) and vitamin K (+154%; $P < 0.001$) were significantly increased after 8 weeks of intervention with vegetables and oil compared with baseline [for details see (29)]. Also plasma folate (+13%; $P = 0.003$) and red blood cell folate (+10%; $P = 0.043$) were significantly increased (Table III). None of the compounds changed significantly in the information group, apart from β-carotene showing a significant increase after 8 weeks (+31%; $P = 0.007$). However, the changes in all the compliance markers were significantly higher in the intervention group compared with the information group. Vitamin B12 was significantly reduced (−9%; $P = 0.003$) in subjects of the intervention group, but changes were not significantly different between the two treatment groups. No effects on homocysteine levels in any of the groups were observed (Table III).

The changes in folate and vitamin B12 levels led to changes in the percentage number of deficient participants (Table II). HbA_{1c} was improved in diabetic individuals in both, the intervention (baseline: 7.58 ± 0.93%, week 8: 7.36 ± 0.80) and information group (baseline: 7.48 ± 0.80%, week 8: 7.25 ± 0.89%), while no changes were observed in non-diabetic individuals.

The results of the CBMN assay are shown in Table IV. No changes in MN, NBud or NPB frequency in the information or intervention group were observed. There was a significant treatment × health interaction ($P = 0.015$) with respect to the changes in NBuds. The intervention with vegetables and plant oil led to a significant increase in apoptosis. There was no effect on necrosis and NDI.

Discussion

The number of people suffering from diabetes is increasing (1) as well as the evidence for a positive association between T2DM and both, cancer (4) and CVD risk (37,38). Life style and especially diet is suggested to affect the onset and progression of the above-mentioned diseases (39,40). Therefore, we designed this human intervention study (i) to assess genome stability, measured by the CBMN assay, in diabetic and non-diabetic individuals and (ii) to evaluate the impact of a healthy diet rich in vegetables and PUFA-rich plant oil as a replacement of SFA on chromosomal damage.

Within this trial, levels of chromosomal damage were not significantly different between diabetic and non-diabetic

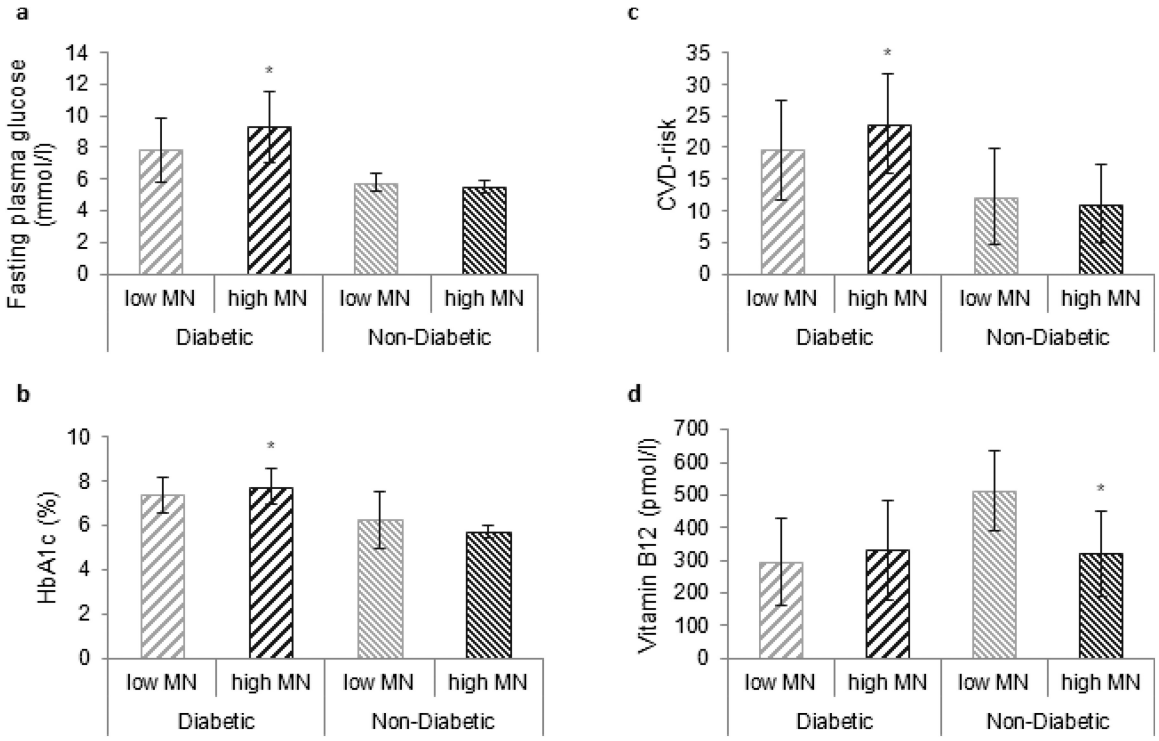


Fig. 1. Comparison of (**a**) fasting plasma glucose (mmol/l), (**b**) glycosylated haemoglobin (HbA_{1c}) (%), (**c**) cardiovascular disease (CVD) risk and (**d**) plasma vitamin B12 concentrations (pmol/l) in diabetic and non-diabetic subjects in the low and high 50th percentile of gender adjusted MN frequency at baseline. *Significantly different from the low 50th percentile of the corresponding group.

individuals. This finding is in contrast to a case control study reporting higher MN frequency in diabetic individuals (16). However, it is worth mentioning that diabetic patients of the Mexican cohort had rather high HbA_{1c} (9.44 ± 1.88%) and fasting plasma glucose levels (209.72 ± 78.11 mg/dl, equal to 11.64 ± 4.34 mmol/l), indicating poor glycaemic control, compared with the well-controlled diabetic patients of this trial. The significant correlation between MN frequency and fasting plasma glucose levels suggests increased chromosomal damage, manifested as increased MN frequency, with worsening of glycaemic control. The importance of proper blood glucose control could also be confirmed by the significantly lower levels of fasting plasma glucose and HbA_{1c} levels in diabetic subjects in the low 50th percentile of MN frequency relative to those in the high 50th percentile.

There is growing evidence that DNA damage is also present in CVD (18,41) and that MN frequency is associated prospectively with increased cardiovascular disease mortality (42). Furthermore, Andreassi *et al.* (17) suggested diabetes beside chronic nitrate treatment as a major independent determinant of increased MN frequency in patients with coronary artery disease. Within this trial, we found increased CVD risk (assessed by the Framingham general cardiovascular risk score) in diabetic individuals with high MN frequency compared with those with low MN frequency.

Another important modulating factor regarding chromosomal stability is micronutrient intake. Folate plays a key role within the DNA methylation and DNA biosynthesis pathways (43). In case of folate as well as vitamin B12 deficiency, uracil is incorporated into DNA. Excessive uracil incorporation leads

Table III. Changes in vitamin B12, folate and homocysteine after 8 weeks in the intervention and information group

	Information			Intervention		
	Baseline	Week 8	P^a	Baseline	Week 8	P^a
Vitamin B12 (pmol/l)			0.230			0.003
Diabetic	302 ± 125	307 ± 144		312 ± 146	290 ± 140	
Non-diabetic	434 ± 178	416 ± 142		384 ± 136	322 ± 129	
Plasma folate (nmol/l)			0.817			0.003
Diabetic	17.4 ± 8.82	17.4 ± 11.2		20. ± 9.02	22.5 ± 9.46	
Non-diabetic	19.5 ± 7.55	23.0 ± 9.43		23.7 ± 5.43	24.5 ± 6.14	
Red blood cell folate (nmol/l)			0.311			0.043
Diabetic	348 ± 190	311 ± 191		346 ± 253	418 ± 293	
Non-diabetic	236 ± 86.0	241 ± 95.1		387 ± 137	414 ± 130	
Homocysteine (μmol/l)			0.067			0.321
Diabetic	13.0 ± 3.06	12.4 ± 3.35		12.4 ± 2.82	12.1 ± 2.77	
Non-diabetic	13.2 ± 3.94	12.8 ± 4.01		11.2 ± 2.25	11.3 ± 2.07	

Data are presented as means ± SD.
[a]*P* values refer to differences between baseline and week 8 in the information and intervention group and were calculated with univariate ANOVA.

Table IV. Changes in CBMN assay parameters after 8 weeks in the intervention and information group

	Information			Intervention		
	Baseline	Week 8	P^a	Baseline	Week 8	P^a
BN with MN per 1000 BN			0.871[b]			0.516[b]
Diabetic	23.0 ± 8.51	23.7 ± 10.8		20.7 ± 7.44	22.5 ± 9.35	
Non-diabetic	22.5 ± 4.68	21.8 ± 5.05		23.3 ± 11.3	24.2 ± 14.9	
BN with NPB per 1000 BN			0.204			0.236
Diabetic	1.9 ± 1.48	2.12 ± 1.64		1.56 ± 1.24	1.85 ± 1.39	
Non-diabetic	1.44 ± 0.62	2.06 ± 0.95		2.01 ± 1.36	2.42 ± 1.41	
BN with NBuds per 1000 BN			0.900			0.152
Diabetic	5.08 ± 2.97	4.73 ± 2.33		3.92 ± 2.03	5.01 ± 2.66	
Non-diabetic	2.69 ± 1.81	3.22 ± 1.95		6.38 ± 5.02	5.47 ± 3.85	
Apoptosis			0.939			0.006
Diabetic	1.44 ± 0.69	1.28 ± 0.47		1.2 ± 0.5	1.47 ± 0.56	
Non-diabetic	1.14 ± 0.37	1.42 ± 0.6		1.46 ± 0.49	1.46 ± 0.49	
Necrosis			0.270			0.569
Diabetic	1.28 ± 0.75	1.29 ± 0.46		1.41 ± 0.76	1.47 ± 0.67	
Non-diabetic	0.95 ± 0.51	1.03 ± 0.42		1.56 ± 0.81	1.23 ± 0.41	
NDI			0.863			0.367
Diabetic	1.96 ± 0.14	1.95 ± 0.15		1.92 ± 0.14	1.94 ± 0.14	
Non-diabetic	2.04 ± 0.06	2.04 ± 0.11		1.92 ± 0.15	1.92 ± 0.18	

Data are presented as means ± SD; BN, binucleated cells; MN, micronuclei; NBud, nuclear bud; NDI, nuclear division index; NPB, nucleoplasmic bridge.
[a]*P* values refer to differences between baseline and week 8 in the information and intervention group and were calculated with univariate ANOVA.
[b]Adjusted for sex.

to point mutations resulting in DNA strand breaks, chromosome breakage and MN formation (44). Also antioxidants are needed for the inactivation of free radicals to prevent interactions with the DNA backbone, leading to DNA strand breaks and MN formation [for review see (22,23)].

Within this trial, plasma and erythrocyte folate concentrations were significantly increased after 8 weeks of vegetable and oil intervention. However, biomarkers for chromosomal damage remained unchanged, only apoptosis was significantly increased. Levels of vitamin B12 were reduced after the intervention, probably due to a replacement of meat and meat products by vegetables. A decreased consumption of animal products was also confirmed by FFQ, which were collected at baseline and week 16 (8 weeks after end of intervention; data not shown).

Based on an intervention study with folate and vitamin B12, Fenech *et al*. (35,44) suggest red blood cell folate concentrations >700 nmol/l, plasma vitamin B12 >300 pmol/l and homocysteine levels <7.5 µmol/l to minimise MN formation. Even though folic acid was increased, only 6% of participants assigned to the intervention group had red blood cell folate levels >700 nmol/l. The required homocysteine and B12 levels were reached by 2% and 26% of subjects of the intervention group, respectively. The vitamin B12 threshold of 300 pmol/l to minimise MN formation could be confirmed within this study, since non-diabetic individuals in the high 50th percentile of MN frequency had significantly lower vitamin B12 plasma levels (320 ± 130 vs. 512 ± 121 pmol/l).

Based on an Australian dietary intake survey (24), apart from folate, calcium, nicotinic acid, vitamin E, retinol (reduction in MN frequency in the tertile with high intake), pantothenic acid, biotin and riboflavin (increase in MN frequency in the tertile with high intake) also were identified as having strong impact on genome health. For β-carotene, a U-shaped dose–response for dietary intake with high MN frequency in the high and low tertile was observed.

Human intervention trials with single antioxidants such as vitamin C (2 g) (45) or vitamin E (50 mg as cereals followed by 335 mg as of soy bean oil, each for 8 weeks) observed either no effect on MN frequency or the obtained results were not conclusive since MN frequency was reduced in both, the control and intervention group (46). The authors concluded that vitamin E is unlikely to affect chromosomal damage due to its activity only in lipid phases and the probably low increase of vitamin E in nuclear membranes, which are surrounding the DNA. Intervention studies with multiple antioxidants supplementing 1000 mg ascorbic acid and 335.5 mg vitamin E (47), a mixed supplement consisting of 100 mg α-tocopherol, 6 mg β-carotene, 100 mg vitamin C and 50 µg selenium (26), or a vitamin–antioxidant mixture containing 3 mg vitamin A, 30 mg α-tocopherol, 150 mg ascorbic acid, 15 mg β-carotene, 0.2 mg folic acid and 75 mg rutin (25) reported positive effects on MN frequency.

In this trial, no supplements were administered. Antioxidant and vitamin concentrations were increased by providing natural foods. Therefore, we hypothesise that a reason for not observing any change in genome damage could be the 'lower administered dose' of vitamins and antioxidants compared with the above-mentioned trials. Only apoptosis was significantly increased during the intervention. However, considering the percentage of apoptotic cells, this increase might not be of biological significance. The results obtained within this trial were in the normal range (33) and comparable to other recent studies of the Austrian population (48,49).

There are, however, two limitations in the present study. First, an intervention period of 8 weeks is at the lower end to detect changes in levels of genome damage in PBMCs, since their half-life is longer (50). Second, due to the modest number of healthy participants, effects of the intervention were evaluated together, in diabetic and non-diabetic individuals. Anyhow, the number of participants in the information and intervention group was big enough to detect changes of biological significance.

In conclusion, T2DM patients with well-controlled glucose metabolism do not have higher rates of genome damage compared with healthy individuals. However, glycaemic control, CVD risk and vitamin B12 could be identified as important factors being associated with MN frequency. The intervention with 300 g

vegetables and 25 ml PUFA-rich plant oil was found to have no impact on levels of MN, NBud and NPB frequency. Based on the measured concentrations of vitamins and antioxidants, we conclude that it is unlikely to reach concentrations shown to protect from genome damage via a daily consumption of 300 g vegetables and 25 ml plant oil for 8 weeks.

Supplementary data

Supplementary Table I is available at *Mutagenesis* Online.

Funding

This work was supported by European Union, through the cross-border cooperation programme Slovakia – Austria 2007–2013 (N00039), and the Austrian Ministry of Health.

Acknowledgements

The authors thank all the staff of the diabetes clinic at the Health Centre South, especially Dr Martina Sonnenfeld and Dr Wolfgang Waldschütz, for their support during the recruitment process, and the head of the laboratory at the health centre, Dr Ernst Forster, for his valuable support. Furthermore, we acknowledge Yvonne Millner, Kristina Paller for performing the vitamin and fatty acid analysis and Theresia Fastian for her help and support throughout this trial. We are grateful to IGLO, Austria, for providing the vegetables.

Conflict of interest statement: The authors declare no conflict of interest.

References

1. Whiting, D. R., Guariguata, L., Weil, C. and Shaw, J. (2011) IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes Res. Clin. Pract.*, **94**, 311–321.
2. Hu, G., Qiao, Q. and Tuomilehto, J. (2001) Glucose tolerance and cardiovascular mortality. *Cardiovasc. Rev. Rep.*, **22**, 649–654, 692.
3. Stamler, J., Vaccaro, O., Neaton, J. D. and Wentworth, D. (1993) Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the multiple risk factor intervention trial. *Diabetes Care*, **16**, 434–444.
4. Grote, V. A., Becker, S. and Kaaks, R. (2010) Diabetes mellitus type 2—an independent risk factor for cancer? *Exp. Clin. Endocrinol. Diabetes*, **118**, 4–8.
5. Castillo, J. J., Mull, N., Reagan, J. L., Nemr, S. and Mitri, J. (2012) Increased incidence of non-Hodgkin lymphoma, leukemia, and myeloma in patients with diabetes mellitus type 2: a meta-analysis of observational studies. *Blood*, **119**, 4845–4850.
6. Wang, C., Wang, X., Gong, G., Ben, Q., Qiu, W., Chen, Y., Li, G. and Wang, L. (2012) Increased risk of hepatocellular carcinoma in patients with diabetes mellitus: a systematic review and meta-analysis of cohort studies. *Int. J. Cancer*, **130**, 1639–1648.
7. Ben, Q., Xu, M., Ning, X., Liu, J., Hong, S., Huang, W., Zhang, H. and Li, Z. (2011) Diabetes mellitus and risk of pancreatic cancer: a meta-analysis of cohort studies. *Eur. J. Cancer*, **47**, 1928–1937.
8. Jiang, Y., Ben, Q., Shen, H., Lu, W., Zhang, Y. and Zhu, J. (2011) Diabetes mellitus and incidence and mortality of colorectal cancer: a systematic review and meta-analysis of cohort studies. *Eur. J. Epidemiol.*, **26**, 863–876.
9. Yuhara, H., Steinmaus, C., Cohen, S. E., Corley, D. A., Tei, Y. and Buffler, P. A. (2011) Is diabetes mellitus an independent risk factor for colon cancer and rectal cancer? *Am. J. Gastroenterol.*, **106**, 1911–1921.
10. Miao Jonasson, J., Cederholm, J., Eliasson, B., Zethelius, B., Eeg-Olofsson, K. and Gudbjörnsdottir, S. (2012) HbA1C and cancer risk in patients with type 2 diabetes—a nationwide population-based prospective cohort study in Sweden. *PLoS ONE*, **7**, e38784.
11. Giovannucci, E., Harlan, D. M., Archer, M. C., Bergenstal, R. M., Gapstur, S. M., Habel, L. A., Pollak, M., Regensteiner, J. G. and Yee, D. (2010) Diabetes and cancer: a consensus report. *Diabetes Care*, **33**, 1674–1685.
12. Kowall, B., Rathmann, W., Strassburger, K., Heier, M., Holle, R., Thorand, B., Giani, G., Peters, A. and Meisinger, C. (2010) Association of passive and active smoking with incident type 2 diabetes mellitus in the elderly population: the KORA S4/F4 cohort study. *Eur. J. Epidemiol.*, **25**, 393–402.
13. Stratton, M. R., Campbell, P. J. and Futreal, P. A. (2009) The cancer genome. *Nature*, **458**, 719–724.
14. Bonassi, S., Znaor, A., Ceppi, M. *et al.* (2007) An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis*, **28**, 625–631.
15. Shettigar, S. K. G., Shailaja, C. and Kulkarni, R. K. (2012) Elevated micronuclei frequency in type 2 diabetes with high glycosylated hemoglobin. *Diabetes Res. Clin. Pract.*, **95**, 246–250.
16. Martínez-Pérez, L. M., Cerda-Flores, R. M., Gallegos-Cabriales, E. C., Dávila-Rodríguez, M. I., Ibarra-Costilla, E. and Cortés-Gutiérrez, E. I. (2007) Frequency of micronuclei in Mexicans with type 2 diabetes mellitus. *Prague Med. Rep.*, **108**, 248–255.
17. Andreassi, M. G., Botto, N., Simi, S., Casella, M., Manfredi, S., Lucarelli, M., Venneri, L., Biagini, A. and Picano, E. (2005) Diabetes and chronic nitrate therapy as co-determinants of somatic DNA damage in patients with coronary artery disease. *J. Mol. Med.*, **83**, 279–286.
18. Botto, N., Rizza, A., Colombo, M. G., Mazzone, A. M., Manfredi, S., Masetti, S., Clerico, A., Biagini, A. and Andreassi, M. G. (2001) Evidence for DNA damage in patients with coronary artery disease. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.*, **493**, 23–30.
19. Guven, M., Guven, G. S., Oz, E., Ozaydin, A., Batar, B., Ulutin, T., Hacihanefioglu, S. and Domanic, N. (2007) DNA repair gene XRCC1 and XPD polymorphisms and their association with coronary artery disease risks and micronucleus frequency. *Heart Vessels*, **22**, 355–360.
20. Bull, C. F., Beetstra-Hill, S., Benassi-Evans, B. J., Crott, J. W., Kimura, M., Teo, T., Wu, J. and Fenech, M. F. (2011) Application and adaptation of the in vitro micronucleus assay for the assessment of nutritional requirements of cells for DNA damage prevention. *Mutagenesis*, **26**, 193–197.
21. Fenech, M. (2008) Genome health nutrigenomics and nutrigenetics—diagnosis and nutritional treatment of genome damage on an individual basis. *Food Chem. Toxicol.*, **46**, 1365–1370.
22. Fenech, M. F. (2010) Dietary reference values of individual micronutrients and nutriomes for genome damage prevention: current status and a road map to the future. *Am. J. Clin. Nutr.*, **91**, 1438S–1454S.
23. Thomas, P., Wu, J., Dhillon, V. and Fenech, M. (2011) Effect of dietary intervention on human micronucleus frequency in lymphocytes and buccal cells. *Mutagenesis*, **26**, 69–76.
24. Fenech, M., Baghurst, P., Luderer, W., Turner, J., Record, S., Ceppi, M. and Bonassi, S. (2005) Low intake of calcium, folate, nicotinic acid, vitamin E, retinol, β-carotene and high intake of pantothenic acid, biotin and riboflavin are significantly associated with increased genome instability—results from a dietary intake and micronucleus index survey in South Australia. *Carcinogenesis*, **26**, 991–999.
25. Gaziev, A. I., Sologub, G. R., Fomenko, L. A., Zaichkina, S. I., Kosyakova, N. I. and Bradbury, R. J. (1996) Effect of vitamin-antioxidant micronutrients on the frequency of spontaneous and in vitro γ-ray-induced micronuclei in lymphocytes of donors: the age factor. *Carcinogenesis*, **17**, 493–499.
26. Smolková, B., Dušinská, M., Rašlová, K., Barančoková, M., Kažimírová, A., Horská, A., Spustová, V. and Collins, A. (2004) Folate levels determine effect of antioxidant supplementation on micronuclei in subjects with cardiovascular risk. *Mutagenesis*, **19**, 469–476.
27. D'Agostino, R. B., Sr, Vasan, R. S., Pencina, M. J., Wolf, P. A., Cobain, M., Massaro, J. M. and Kannel, W. B. (2008) General cardiovascular risk profile for use in primary care: the Framingham heart study. *Circulation*, **117**, 743–753.
28. American Diabetes Association (2009) Diagnosis and classification of diabetes mellitus. *Diabetes Care*, **32**, S62–S67.
29. Müllner, E., Brath, H., Pleifer, S., *et al.* (2012) Vegetables and PUFA-rich plant oil reduce DNA strand breaks in individuals with type 2 diabetes. *Mol. Nutr. Food Res*. DOI 10.1002/mnfr.201200343.
30. Wagner, K. H., Auer, E., and Elmadfa, I. (2000) Content of trans fatty acids in margarines, plant oils, fried products and chocolate spreads in Austria. *Eur. Food Res. Technol.*, **210**, 237–241.
31. Jakob, E. and Elmadfa, I. (1995) Rapid HPLC assay for the assessment of vitamin K1, A, E and beta-carotene status in children (7–19 years). *Int. J. Vitam. Nutr. Res.*, **65**, 31–35.
32. Majchrzak, D., Singer, I., Männer, M., Rust, P., Genser, D., Wagner, K. H. and Elmadfa, I. (2007) B-vitamin status and concentrations of homocysteine in Austrian omnivores, vegetarians and vegans. *Ann. Nutr. Metab.*, **50**, 485–491.
33. Fenech, M. (2007) Cytokinesis-block micronucleus cytome assay. *Nat. Protocol.*, **2**, 1084–1104.
34. Fenech, M., Chang, W. P., Kirsch-Volders, M., Holland, N., Bonassi, S., and Zeiger, E. (2003) HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.*, **534**, 65–75.

35. Fenech, M., Aitken, C. and Rinaldi, J. (1998) Folate, vitamin B12, homocysteine status and DNA damage in young Australian adults. *Carcinogenesis*, **19**, 1163–1171.
36. Fenech, M. and Morley, A. A. (1986) Cytokinesis-block micronucleus method in human lymphocytes: effect of in vivo ageing and low dose X-irradiation. *Mutat. Res.*, **161**, 193–198.
37. Eeg-Olofsson, K., Cederholm, J., Nilsson, P. M., Zethelius, B., Nunez, L., Gudbjörnsdóttir, S. and Eliasson, B. (2009) Risk of cardiovascular disease and mortality in overweight and obese patients with type 2 diabetes: an observational study in 13,087 patients. *Diabetologia*, **52**, 65–73.
38. Hu, G., Jousilahti, P., Qiao, Q., Katoh, S. and Tuomilehto, J. (2005) Sex differences in cardiovascular and total mortality among diabetic and non-diabetic individuals with or without history of myocardial infarction. *Diabetologia*, **48**, 856–861.
39. Buttar, H. S., Li, T. and Ravi, N. (2005) Prevention of cardiovascular diseases: role of exercise, dietary interventions, obesity and smoking cessation. *Exp. Clin. Cardiol.*, **10**, 229–249.
40. Uauy, R. and Solomons, N. (2005) Diet, nutrition, and the life-course approach to cancer prevention. *J. Nutr.*, **135**, 2934S–2945S.
41. Matturri, L., Cazzullo, A., Turconi, P., Lavezzi, A. M., Vandone, P. L., Gabrielli, L., Fernández Alonso, G., Grana, D. and Milei, J. (2001) Chromosomal alterations in atherosclerotic plaques. *Atherosclerosis*, **154**, 755–761.
42. Murgia, E., Maggini, V., Barale, R. and Rossi, A. M. (2007) Micronuclei, genetic polymorphisms and cardiovascular disease mortality in a nested case-control study in Italy. *Mutat. Res. Fund. Mol. Mech. Mutagen.*, **621**, 113–118.
43. Lamprecht, S. A. and Lipkin, M. (2003) Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. *Nat. Rev. Cancer*, **3**, 601–614.
44. Fenech, M. (2012) Folate (vitamin B9) and vitamin B12 and their function in the maintenance of nuclear and mitochondrial genome integrity. *Mutat. Res. Fund. Mol. Mech. Mutagen.*, **733**, 21–33.
45. Crott, J. W. and Fenech, M. (1999) Effect of vitamin C supplementation on chromosome damage, apoptosis and necrosis ex vivo. *Carcinogenesis*, **20**, 1035–1041.
46. Fenech, M., Dreosti, I. and Aitken, C. (1997) Vitamin-E supplements and their effect on vitamin-E status in blood and genetic damage rate in peripheral blood lymphocytes. *Carcinogenesis*, **18**, 359–364.
47. Schneider, M., Diemer, K., Engelhart, K., Zankl, H., Trommer, W. E. and Biesalski, H. K. (2001) Protective effects of vitamins C and E on the number of micronuclei in lymphocytes in smokers and their role in ascorbate free radical formation in plasma. *Free Radic. Res.*, **34**, 209–219.
48. Reichhold, S., Neubauer, O., Ehrlich, V., Knasmüller, S., and Wagner, K. H. (2008) No acute and persistent DNA damage after an ironman triathlon. *Cancer Epidemiol. Biomarkers Prev.*, **17**, 1913–1919.
49. Wallner, M., Blassnigg, S. M., Marisch, K., *et al.* (2012) Effects of unconjugated bilirubin on chromosomal damage in individuals with Gilbert's syndrome measured with the micronucleus cytome assay. *Mutagenesis*, **27**, 731–735.
50. Ramalho, A. T., Curado, M. P. and Natarajan, A. T. (1995) Lifespan of human lymphocytes estimated during a six year cytogenetic follow-up of individuals accidentally exposed in the 1987 radiological accident in Brazil. *Mutat. Res. Fund. Mol. Mech. Mutagen.*, **331**, 47–54.

Supplementary Table 1. Calculated composition of the intervention vegetables per 300 g (equivalent to daily portion size)

	Energy (kJ)	Carotene (mg)	Folate (µg)	Vitamin C (mg)	Vitamin E (mg TE)
Strained spinach	246	15	282	120	4.4
Green beans	576	0.8	78	33	0.3
Broccoli	372	2.7	78	217	2.0
Brussels sprouts	441	1.4	189	248	1.7
Soybeans	1803	1.2	132	87	0.9
Peas	1068	1.3	315	53	0.2
Carrots[1]	729	27	27	8	1.3
Romaine lettuce with peas	775	1.4	56	14	7.0
Leave spinach	246	15	282	120	4.4
Vegetable mix I (broccoli, cauliflower)	313	1.1	224	263	1.1
Vegetable mix II (broccoli, carrot yellow and orange)	305	17	90	96	1.7
Vegetable mix III (carrot, potato, kohlrabi, leek, pea)	632	6.0	89	79	1.0
Vegetable mix IV (pea, carrot, corn)	738	8.7	91	46	0.6
Vegetable mix V (carrot, potato, broccoli, green bean, cauliflower)	583	5.7	124	125	0.8
Roasted vegetables I (carrot, broccoli, cauliflower, pole beans, zucchini)[2]	614	6.1	130	127	1.0
Roasted vegetables II (carrot, Brussels sprouts, kohlrabi, pole beans)[2]	751	7.1	119	148	1.3
Ready meal: Spinach with potatoes[3]	1239	9.1	169	80	4.7

[1] was given to subjects additionally, in case of any digestive discomfort; [2] Subjects got roasted vegetables I for week 1, 2, 5, 6 and roasted vegetables II for week3, 4, 7 and 8; [3] Contains 230 g of spinach.

III

Nuclear anomalies in exfoliated buccal cells in healthy and diabetic individuals and the impact of a dietary intervention

Elisabeth Müllner[1*], Helmut Brath[2], Armen Nersesyan[3], Marlies Nitz[1], Alice Petschnig[1], Marlies Wallner[1], Siegfried Knasmüller[3], Karl-Heinz Wagner[1]

[1] Department of Nutritional Sciences, Emerging Field "Oxidative Stress and DNA Stability", University of Vienna, Austria

[2] Diabetes Outpatient Clinic, Health Centre South, Vienna, Austria

[3] Medical University of Vienna, Institute of Cancer Research, Vienna, Austria

Abstract

Objective – The purpose of this study was to compare the frequencies of nuclear anomalies in buccal cells between diabetic and non-diabetic individuals and to assess the impact of a "healthy diet" - a cornerstone in the treatment of diabetes.

Research Design and Methods – Seventy six diabetic and 21 non-diabetic individuals participated in this parallel, randomized, intervention trial. All participants received information about the importance of a healthy diet, while participants randomly assigned to the intervention group received additionally 300 g of vegetables and 25 ml plant oil rich in polyunsaturated fatty acids (PUFA) per day for 8 weeks. Cytogenetic damage in buccal cells was assessed at baseline and after 8 weeks using the buccal micronucleus cytome assay.

Results – Micronucleus (MN) frequency at baseline was significantly higher in participants with diabetes (0.58±0.30‰) compared with non-diabetic individuals (0.28±0.29‰). Further analysis of baseline data revealed significantly higher MN levels in participants of the highest tertile of waist circumference (+40%), fasting plasma glucose (+55%), glycated hemoglobin (+41%) and cardiovascular disease risks (+39%) relative to participants of the lowest tertile. No associations between folic acid, vitamin B12, homocysteine and MN frequencies were observed. The dietary intervention had no effect on MN frequencies and other biomarkers for chromosomal stability.

Conclusions – The results of this study suggest a strong impact of abdominal obesity and glucose metabolism on genomic stability. However, a dietary intervention with vegetables and PUFA-rich plant oil had no protective effects.

*To whom correspondence should be addressed:
Elisabeth Müllner
University of Vienna, Department of Nutritional Sciences
Althanstraße 14, 1090 Vienna, Austria
E-mail: elisabeth.muellner@univie.ac.at

Introduction

Type 2 diabetes is one of the most common chronic diseases and a growing health care problem worldwide. It is well recognized that it is linked to increased risk of micro- and macrovascular complications (1). Vigneri et al. suggest in a review associations between type 2 diabetes and risk of liver, pancreatic, colorectal, bladder, kidney cancer as well as non-Hodgkin's Lymphoma (2).

Several factors, like obesity, quality of metabolic control, medical treatment and lifestyle have a major impact on the onset of diabetes-associated complications. Especially for cancer, it is not clear yet, if the association is direct or mediated via common predisposing risk factors such as aging, obesity, physical inactivity or poor diet quality (3). Hypothesized mechanisms for increased cancer risk in patients with type 2 diabetes include hyperglycemia, insulin resistance and hyperinsulinemia (4).

Genomic instability and damage is a central event in the development of many diseases, including cancer (5). A biomarker reflecting genomic damage is the micronucleus (MN) (frequency), representing the loss of a whole chromosome or fragments (6) and it was postulated that the assessment of MNi in exfoliated buccal cells, a non-invasive method, might be of high relevance in future as up to 90% of all cancers are of epithelial origin (7).

Zúñiga-González et al. (8) found significantly increased MN levels in exfoliated buccal cells in diabetic patients (type 1 and type 2) compared to healthy individuals and showed a reduction in MN frequency after high dose of folic acid supplementation (15 mg/d). The effect of natural foods on genomic stability in diabetic individuals has not been investigated yet, although beneficial effects of a healthy diet are supported by inverse association between fruit consumption and buccal MN frequency (9), and intake of fruits, vegetables and cruciferous vegetables and cancer risk (10).

In order to compare DNA integrity between diabetic and non-diabetic individuals and to assess the impact of a healthy diet, realized through the replacement of saturated fatty acids (SFA) by polyunsaturated fatty acids (PUFA) and intake of 300 g of vegetables per day, a human intervention study was conducted. Apart of MN levels, frequencies of nuclear buds (or broken eggs (BE)), indicative for gene amplification and binucleated cells (BNC), reflecting cytokinetic defects, were monitored. Furthermore, karyolytic (KL), pycnotic (PC), karyorrhectic (KR) cells and cells with condensed chromatin (CC) which reflect acute cell death (6) were scored. All these parameters are very novel and have not been investigated in a natural food based intervention.

Research Design and Methods

Study population

Patients with type 2 diabetes (36 treated with insulin, 40 treated with oral antidiabetic drugs) were recruited from a local diabetes clinic (Diabetes Outpatient Clinic, Health Centre South, Vienna, Austria). Non-diabetic individuals (n=21) were partners of the diabetic subjects.

Individuals with type 2 diabetes had to have stable metabolic control (constant medication regarding glucose, lipid and uric acid metabolism), glycated hemoglobin (HbA1c) concentration <9.5% (80 mmol/mol), serum total cholesterol (TC) <300 mg/dl (<7.76 mmol/l), serum triglycerides (TG) <500 mg/dl (<5.7 mmol/l) and serum creatinine <2.5 mg/dl (<221 µmol/l). Non-diabetic individuals had to be free of diabetes and were not allowed to take glucose lowering drugs. Otherwise the same inclusion criteria were applied.

All subjects had stable body weights, constant dietary habits and physical activity levels for at least four weeks before entry to the study. Subjects who intended to change dietary habits, frequency of physical activity or body weight within the study period were excluded from the participation. Further exclusion criteria were smoking, intake of supplements containing fish oil or other fatty acids. The medical therapies of participants were not changed during the study.

The study protocol was approved by the Ethical Committee of the City of Vienna (EK09-218-VK_NZ) and the trial was registered on Current Controlled Trials (ISRCTN53451803). The inclusion criteria were fulfilled by 151 subjects. 120 gave their written consent. Out of these, 21 withdrew because of health problems unrelated to the study, digestive discomfort or scheduling conflicts. 2 subjects, reported not having diabetes, were excluded because of increased fasting glucose levels.

Study design and dietary intervention

All participants (diabetic and non-diabetic individuals) received information about the beneficial effects of a healthy diet with special focus on the importance of fat quality and the role of vegetables in a balanced diet. Participants were randomly assigned to the “intervention” or “information only” group. Subjects of the “information only” group received the above mentioned information, while subjects of the “intervention” group consumed additionally 25 ml of PUFA-rich walnut-oil (SFA:MUFA:PUFA: 9.3:17.4:73.3) per day and

300 g vegetables. A variety of frozen vegetables (spinach, green beans, broccoli, Brussels sprouts, soybeans, peas, carrots, romaine lettuce with peas, different vegetable mixes with broccoli, cauliflower, carrots, kohlrabi, leek, peas, corn, zucchini or pole beans; for details see (11; 12)) was given to the participants. The participants were instructed to use the plant oil as replacement for SFA. A reference "cup" and a booklet with recipes, instructions for replacement of SFA and usage of the plant oil (oil was not allowed to be heated up, but added to warm foods) was provided to the participants. A dietary diary had to be completed, and fatty acid profile and various vitamin concentrations were measured to monitor compliance.

The intervention period lasted 8 weeks, followed by a period of 8 weeks in which no intervention foods were provided. Blood samples were taken before the intervention, after 4, 8 (end of intervention period) and 16 weeks. The buccal micronucleus cytome (BMCyt) assay was performed at baseline and week 8.

Anthropometric and blood pressure measurements and Framingham general cardiovascular risk

For all anthropometric measurements participants were lightly dressed, without wearing shoes. Body height (stadiometer: Seca, Modell 214, Hamburg, Germany), body weight (digital scale: Seca, Bella 840, Hamburg, Germany) and waist circumference were measured. Body mass index (BMI) was calculated as kg/m^2.

At each study visit, three blood pressure measurements (BpTRU Medical Devices, Coquitlam, BC, Canada) with 2 min intervals in between (the mean of the last two measurements was used) were obtained after at least 5 min rest with the subject in a seated position. The Framingham general cardiovascular risk (13) was estimated, using following variables: age, sex, tobacco use, treated/untreated systolic blood pressure, diabetes and TC, HDL-cholesterol.

Blood sampling and laboratory analysis

Venous blood samples were obtained after an overnight fast using heparin or serum tubes (Becton Dickinson, Schwechat, Austria). After centrifugation serum and plasma were aliquoted, used fresh or frozen at -80°C for further analysis. Erythrocytes were washed three times with isotonic phosphate buffer, aliquoted and stored at -80°C.

Fasting plasma glucose, HbA1c, high-density lipoprotein (HDL), TC and insulin were measured immediately by the laboratory of the Health Centre South, Vienna. HbA1c was analyzed in whole blood by HPLC (automated Glycohemoglobin Analyzer HLC-723G8; Tosoh, Tokyo, Japan). Fasting plasma glucose was determined enzymatically by the hexokinase method (Aeroset, Abbott Diagnostics, Illinois, USA), plasma insulin concentrations were measured on an Immulite 2000 immunochemistry system (Siemens Medical Solutions Diagnostics, Flanders, USA). Homeostasis model assessment (HOMA) of insulin resistance was calculated as the product of fasting plasma glucose (mmol/L) and insulin (μU/ml) concentrations, divided by 22.5. Serum TC and HDL cholesterol levels were measured enzymatically by an automated method (Aeroset, Abbott Laboratories, North Chicago, IL, USA) using commercial kits (Abbott).

The fatty acid profile in plasma was determined by a gas chromatograph equipped with a flame ionization detector (14). Identification of fatty acids was based on the comparison of the samples' retention times to those of a 37 Component FAME Mix standard (Supelco, Bellefonte, USA). TotalChrom Workstation 6.3.0, PE Nelson, Perkin Elmer was used for peak integration.

Plasma concentrations of vitamin K, α- and γ-tocopherol, lutein, α- and β-carotene were determined by reverse-phase HPLC (15). Each vitamin was quantitated on the basis of peak area using a calibration curve generated from standard solutions.

Plasma concentrations of vitamin B12 and folic acid were measured according to routine diagnostic tests on Siemens Immulite 2000 analyzer (Siemens Healthcare Diagnostics, Tarrytown, USA) at the laboratory of the Health Centre South, Vienna. Folic acid in erythrocytes was measured using radioimmunoassay (MP Biomedicals, Germany).

Plasma homocysteine was measured by reversed-phase HPLC with a fluorescence detector (emission wavelength: 515 nm; excitation wavelength: 385 nm) on a LiChrosphere column (5 μm, 125 x 4 mm; Merck, Hitachi, LaChrom, Austria). Potassium hydrogenphosphate buffer with 4% acetonitrile were used as mobile phase (16).

Adiponectin was measured with a magnetic bead-based assay (Bio-Plex Pro diabetes assay, Bio-Rad Laboratories, Inc.) and a Bio-Plex array reader using Bio-Plex Manager™ Software 4.1.1.

Buccal Micronucleus Cytome assay

Cells were collected with toothbrushes from both cheeks after subjects rinsed their mouths with tap water. Subsequently, slides were prepared, stained and scored according to the method of Thomas *et al.* (6). Cell suspensions were diluted to a concentration of >80,000 cells/ml. 120 µl of cell suspension were transferred to slides by cytocentrifugation (Shandon Cytocentrifuge Cytospine 4) and fixed with cold methanol (80%) for 30 min and ≥2,000 cells/sample were evaluated.

For Feulgen staining, cells were placed in beakers with 5.0 M HCl at room temperature for 15 min, rinsed with distilled water (15 min) and subsequently stained with Schiff's reagent (90 min). Cells were scored under bright field with 400-fold magnification using oil immersion with Eclipse E 600 microscope (Nikon, Tokyo, Japan) and then confirmed as positive under fluorescence. MNi were scored in a combination of both basal and differentiated cells according to the criteria defined by Thomas *et al.* (6). The analyses of the slides were carried out at the Institute of Cancer Research (Vienna, Austria) by two experienced scorers and recorded after consensus.

As biomarkers for genome damage MN cells, total MNi, BNC and BE and for cell death KL, KR+CC, and P were scored. Since KR and CC cells are difficult to discriminate, they were scored together.

Statistical analysis

Statistical analyses were performed using SPSS 20.0 for Windows (SPSS, Chicago, IL, USA). Normal distribution within the data set was tested by histograms and Kolmogorov-Smirnov test. To assess differences between two groups, independent samples T-test (for parametric data) or Mann-Whitney U-test (for non-parametric data) were conducted.

Baseline data were categorized into tertiles and MN frequencies were compared between them (Mann-Whitney-U-Test and correction for multiple comparisons). Chi-square test was used for comparisons of binary variables.

The effect of the intervention was assessed by comparing baseline values with values after 8 weeks (Wilcoxon Test or paired T-test) and by comparing the changes after 8 weeks between the two treatment groups (Mann-Whitney U-Test or independent samples T-test). Pearson correlation and Spearman rank correlation were used to evaluate associations between variables. Results were considered significant at $P < 0.05$.

Results

Baseline data

Baseline characteristics of diabetic and non-diabetic individuals are presented in Table 1. Diabetic individuals had significantly higher BMI and waist circumference, higher levels of fasting blood glucose, HbA1c and HOMA-insulin resistance. MN frequency and total number of MNi were approximately two fold higher in diabetic patients compared to non-diabetic participants (Table 1).

All participants were non-smokers and no significant differences between the two groups regarding smoking behavior in the past were observed. Levels of genomic damage were not significantly different between ex-smokers (n=49; MN cells: 0.57±0.37‰) and never-smokers (n=48; MN cells: 0.46±0.25‰). However, smoking duration (r = 0.305, *P* = 0.033) and number of cigarettes smoked per day (r = 0.354, *P* = 0.013) were significantly correlated with the MN frequency among ex-smokers. Sex had no impact on genome damage rates (male, MN cells: 0.55±0.27‰; female, MN cells: 0.50±0.35‰).

Analysis of the relationships between clinical chemistry parameters, vitamin concentrations and MN frequency revealed significant associations (Table 2). Participants in the highest tertile of waist circumference, fasting plasma glucose, HbA1c and cardiovascular disease (CVD) risk had significantly higher MN frequencies (+40%, +55%, +41%, +39%, *P* <0.05, respectively) compared to participants in the lowest tertile. In contrast, a trend toward a protective effect of adiponectin was observed. No associations between vitamin B12, plasma folate, red blood cell folate, homocysteine and MN frequency were observed (data not shown).

At baseline there were no significant differences between information and intervention group, apart from levels of pycnotic cells, which were significantly higher in participants of the information group (Table 3).

Effect of the intervention

Dietary compliance was monitored via dietary diaries, showing an average consumption of 294±28.5 g vegetables per day. Furthermore, oil bottles were collected and weighed and showed an intake of 23.4±4.01 ml plant oil per day (density of oil 0.85 g/cm^3).

Plasma and red blood cell analyses confirmed good dietary compliance, and showed significant increases in lutein (+58%; *P* <0.001), α-carotene (+92%; *P* <0.001), β-carotene (+53%; *P* <0.001), vitamin K (+154%; *P* <0.001), plasma folate (+13%; *P* <0.05), red

blood cell folate (+10%; *P* <0.05), γ-tocopherol (+37%; *P* <0.001), linoleic acid (+13%; *P* <0.001) and linolenic acid (+60%; *P* <0.001) (for details see (11; 12)). Body weight, BMI and waist circumference were not altered during the study, neither in the intervention nor in the information group (data not shown). Glycemic control improved significantly during the course of the study: HbA1c decreased in diabetic individuals after 8 weeks from 7.58±0.93% (59±10.2 mmol/mol) to 7.36±0.80% (57±8.7 mmol/mol) in the intervention and from 7.48±0.80% (58±8.7 mmol/mol) to 7.25±0.89% (56±9.7 mmol/mol) in the information group. Therefore changes in HbA1c were not significantly different between the two treatment groups. No changes in HbA1c levels were observed in non-diabetic individuals (information group, baseline: 5.71±0.28% (39±3.1 mmol/mol), after 8 weeks: 5.73±0.30% (39±3.3 mmol/mol); intervention group, baseline: 5.83±0.24 (40±2.6 mmol/mol), after 8 weeks: 5.79±0.47 (40±5.1 mmol/mol)).

During the course of the study the numbers of BNC and KR + CC were decreased in both, the information and intervention group (Table 4). PC were only reduced in participants of the intervention group and changes after 8 weeks were significantly greater in participants of the intervention group compared to the information group. For all other BMCyt-assay parameters, changes after 8 weeks were not significantly different between the two treatment groups.

Conclusions

The results of this study show that levels of buccal MNi in diabetic individuals are approximately two-fold higher than in non-diabetic participants. MNi in exfoliated buccal cells are a novel and non-invasive biomarker of genomic stability, formed during mitosis in the basal cell layer of the epithelium and represent the loss of chromosome fragments or a whole chromosome that failed to be incorporated in the main nuclei (6). Genomic instability is a hallmark of tumorigenesis (5); also cancer patients showed increased levels of buccal MN frequency (17-20) compared with healthy individuals. Therefore, our results which indicate higher genomic instability in patients with diabetes mellitus compared with non-diabetic participants are consistent with epidemiological data indicating increased cancer risk in individuals with type 2 diabetes (2).

Alcohol intake, smoking and dietary habits are known as major modifiable variables influencing genomic stability in healthy individuals (9). Only few studies concerning the frequencies of buccal MN in diabetic individuals were performed so far and they are either of limited quality (21; 22) or did not address the issue of modifiable risk factors (8; 23).

The present study suggests associations between abdominal obesity, glucose metabolism, CVD-risk and buccal MN frequency, while no associations were observed with nutrients involved in DNA synthesis such as folic acid and vitamin B12 or homocysteine.

The observed link between waist circumference and decreased genomic stability is consistent with findings of epidemiological studies showing that obesity in general (24; 25) and waist circumference in particular (26-28) are associated with genomic instability, manifested as increased risk of site-specific malignancies. One mechanism linking obesity and genome instability is insulin resistance and chronic hyperinsulinemia (29). Hyperinsulinemia leads to reduced hepatic production of insulin-like growth factor binding protein 1 and 2 and thereby to increased levels of free, bioactive insulin-like growth factor-1 (IGF-1) (30). IGF-1 induces several biological actions, like cell proliferation, differentiation and inhibition of apoptosis, which may lead to genomic instability and favor tumor growth (31). A trend ($P< 0.1$, Table 2) for significantly different MN levels between participants in the highest and lowest tertile of insulin or HOMA-insulin resistance was observed in the present study.

Insulin resistance is among other factors connecting abdominal obesity and hyperglycemia. High glucose levels may contribute to decreased genomic stability via

increased cell proliferation, enhancing the risk of genetic errors and the possibility of cancer development (32; 33). Glucose is also used as an energy substrate in tumor cells and might thereby have a direct tumor promoting effect (34). Within this study, participants in the highest tertile of fasting plasma and HbA1c showed significantly higher levels of genomic damage compared with participants in the lowest tertile.

Furthermore, a positive association between genomic instability and CVD risk, assessed by the Framingham general cardiovascular risk score (13), was observed. It was postulated that genomic instability is a relevant contributor to atherosclerosis (35) and that patients at increased risk for CVD (12) or with coronary artery disease (36) have higher MN frequency in lymphocytes. Mercer et al. (35) suggested that persistent DNA damage in plaque cells change the ratio of cell proliferation and apoptosis and promotes thereby the risk of atherosclerosis.

Folate and vitamin B12 are important modulating factors of chromosomal stability due to their role in DNA biosynthesis pathways (37). The MN frequencies in lymphocytes are higher under conditions of folate and B12 deficiencies (38) and intervention studies in diabetic individuals with folate showed significant reductions of buccal MN frequencies (8; 23). However, the administered dose (15 mg) within these trials was 37.5-fold higher than the dietary reference intake (DRI) and exceed the upper safe level for folate intake several folds, which cannot be reached via a dietary intervention with whole foods.

Within the present trial, no associations with folate or vitamin B12 were observed and the vitamin B12, plasma and red blood cell folate status were satisfactory (12). Significant increases in antioxidants and vitamins (11; 12) and reductions in HbA1c (11) in the course of the intervention did not lead to a reduction in buccal MN frequency. On the contrary, levels of BNC and KR+CC were significantly reduced. However, it is unlikely that this reduction is due to the intervention since both markers were reduced in the information group as well. Our findings are in contrast with supplementary trials in healthy individuals, showing reductions in MN levels after supplementation with folate (39), vitamin A, β-carotene and canthaxanthin (40). The association between diabetes and genomic stability is multifactorial and our results suggest that improvements in glycemic control, vitamin and antioxidant status within 8 weeks are not sufficient to improve genomic stability in buccal cells.

In conclusion, the results of this study provide important novel information regarding associations between waist circumference, glucose metabolism and genomic damage. An

intervention with vegetables and walnut oil, which was accepted by the subjects and proven for everyday use, did not lead to improvements of genomic stability; nevertheless our results underline the importance of tight glycemic control and optimum body weight and show that providing participants with information reduces HbA1c as much as a nutritional intervention.

Acknowledgments

This work was supported by European Union, through the cross-border cooperation program Slovakia – Austria 2007–2013 (N00039), and the Austrian Ministry of Health. No potential conflicts of interest relevant to this article were reported.

E.M. was involved in designing the study, performed the statistical analysis and wrote the manuscript. H.B. participated in designing the study, was responsible for recruitment of participants, their medical treatment and edited the manuscript. A.N. performed the BMCyt assay (preparation and staining of slides), was responsible for scoring of slides and reviewed the manuscript. M.N. and A.P. assisted in preparation of slides and conducted lab analysis. M.W. substantially assisted in statistical evaluation of data and reviewed the manuscript. S.K. interpreted data, and provided intellectual input. KH.W. provided design and concept of the study, secured funding, and reviewed the manuscript. He is taking responsibility for the contents of the article.

The authors thank all the staff of the diabetes clinic at the Health Centre South, especially Dr. Martina Sonnenfeld and Dr. Wolfgang Waldschütz, for their support during the recruitment process, and the head of the laboratory at the health center, Dr. Ernst Forster, for his valuable support. Special thanks to Dr. Judith Vanek for providing the walnut oil and IGLO Austria for providing the vegetables. Special thanks also to all the participating subjects without whom this study would not have been possible.

References

1. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414:813-820

2. Vigneri P, Frasca F, Sciacca L, Pandini G, Vigneri R: Diabetes and cancer. Endocrine-Related Cancer 2009;16:1103-1123

3. Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, Pollak M, Regensteiner JG, Yee D: Diabetes and cancer: A consensus report. Diabetes Care 2010;33:1674-1685

4. Johnson JA, Carstensen B, Witte D, Bowker SL, Lipscombe L, Renehan AG: Diabetes and cancer (1): Evaluating the temporal relationship between type 2 diabetes and cancer incidence. Diabetologia 2012;55:1607-1618

5. Shen Z: Genomic instability and cancer: an introduction. Journal of Molecular Cell Biology 2011;3:1-3

6. Thomas P, Holland N, Bolognesi C, Kirsch-Volders M, Bonassi S, Zeiger E, Knasmueller S, Fenech M: Buccal micronucleus cytome assay. Nature Protocols 2009;4:825-837

7. Cairns J: Mutation selection and the natural history of cancer. Nature 1975;255:197-200

8. Zúñiga-González GM, Batista-González CM, Gómez-Meda BC, Ramos-Ibarra ML, Zamora-Perez AL, Muñoz-Magallanes T, Ramos-Valdés C, Gallegos-Arreola MP: Micronuclei in diabetes: Folate supplementation diminishes micronuclei in diabetic patients but not in an animal model. Mutation Research - Genetic Toxicology and Environmental Mutagenesis 2007;634:126-134

9. Bonassi S, Coskun E, Ceppi M, Lando C, Bolognesi C, Burgaz S, Holland N, Kirsh-Volders M, Knasmueller S, Zeiger E, Carnesoltas D, Cavallo D, da Silva J, de Andrade VM, Demircigil GC, Odio AD, Donmez-Altuntas H, Gattas G, Giri A, Giri S, Gómez-Meda B, Gómez-Arroyo S, Hadjidekova V, Haveric A, Kamboj M, Kurteshi K, Martino-Roth MG, Montoya RM, Nersesyan A, Pastor-Benito S, Salvadori DMF, Shaposhnikova A, Stopper H, Thomas P, Torres-Bugarín O, Yadav AS, González GZ, Fenech M: The HUman MicroNucleus project on eXfoLiated buccal cells (HUMN XL): The role of life-style, host factors, occupational exposures, health status, and assay protocol. Mutation Research - Reviews in Mutation Research 2011;728:88-97

10. Genkinger JM, Platz EA, Hoffman SC, Comstock GW, Helzlsouer KJ: Fruit, vegetable, and antioxidant intake and all-cause, cancer, and cardiovascular disease mortality in a community-dwelling population in Washington County, Maryland. American Journal of Epidemiology 2004;160:1223-1233

11. Müllner E, Brath H, Pleifer S, Schiermayr C, Baierl A, Wallner M, Fastian T, Millner Y, Paller K, Henriksen T, Poulsen HE, Forster E, Wagner KH: Vegetables and PUFA-rich plant oil reduce DNA strand breaks in individuals with type 2 diabetes. Molecular Nutrition and Food Research 2012;57:328-338
12. Müllner E, Brath H, Toferer D, Adrigan S, Bulla M-T, Stieglmayer R, Wallner M, Marek R, Wagner K-H: Genome damage in peripheral blood lymphocytes of diabetic and non-diabetic individuals after intervention with vegetables and plant oil. Mutagenesis 2013;28:205-211
13. D'Agostino Sr RB, Vasan RS, Pencina MJ, Wolf PA, Cobain M, Massaro JM, Kannel WB: General cardiovascular risk profile for use in primary care: The Framingham heart study. Circulation 2008;117:743-753
14. Wagner KH, Auer E, Elmadfa I: Content of trans fatty acids in margarines, plant oils, fried products and chocolate spreads in Austria. European Food Research and Technology 2000;210:237-241
15. Jakob E, Elmadfa I: Rapid HPLC assay for the assessment of vitamin K1, A, E and beta-carotene status in children (7-19 years). International Journal for Vitamin and Nutrition Research 1995;65:31-35
16. Majchrzak D, Singer I, Männer M, Rust P, Genser D, Wagner KH, Elmadfa I: B-vitamin status and concentrations of homocysteine in Austrian omnivores, vegetarians and vegans. Annals of Nutrition and Metabolism 2007;50:485-491
17. Burgaz S, Coskun E, Demircigil GC, Kocabas NA, Cetindag F, Sunter O, Edinsel H: Micronucleus frequencies in lymphocytes and buccal epithelial cells from patients having head and neck cancer and their first-degree relatives. Mutagenesis 2011;26:351-356
18. Dey P, Samanta S, Susheilia S: Micronucleus assay in buccal smears of breast carcinoma patients. Diagnostic Cytopathology 2012;40:664-666
19. Dórea LNTM, Meireles JRC, Lessa JPR, Oliveira MC, De Bragancía Pereira CA, Polpo De Campos A, Cerqueira EDMML: Chromosomal damage and apoptosis in exfoliated buccal cells from individuals with oral cancer. International Journal of Dentistry 2012;
20. Nersesyan AK, Vardazaryan NS, Gevorgyan AL, Arutyunyan RM: Micronucleus level in exfoliated buccal mucosa cells of cancer patients. Archive of Oncology 2002;10:35-36
21. Nersesyan AK: Increased frequency of micronuclei in diabetes mellitus patients using pioglitazone and glimepiride in combination. Food and Chemical Toxicology 2011;49:2470

22. Shaik NA, Shaik JP, Ali S, Imran A, Rao DK: Increased frequency of micronuclei in diabetes mellitus patients using pioglitazone and glimepiride in combination. Food and Chemical Toxicology 2010;48:3432-3435
23. Lazalde-Ramos BP, Zamora-Perez AL, Sosa-Macías M, Guerrero-Velázquez C, Zúñiga-González GM: DNA and Oxidative Damages Decrease After Ingestion of Folic Acid in Patients with Type 2 Diabetes. Archives of Medical Research 2012;43:476-481
24. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M: Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. The Lancet 2008;371:569-578
25. Roberts DL, Dive C, Renehan AG: Biological mechanisms linking obesity and cancer risk: New perspectives. Annual Reviews of Medicine 2010;61:301-316
26. Berrington de González A, Spencer EA, Bueno-de-Mesquita HB, Roddam A, Stolzenberg-Solomon R, Halkjær J, Tjønneland A, Overvad K, Clavel-Chapelon F, Boutron-Ruault M-C, Boeing H, Pischon T, Linseisen J, Rohrmann S, Trichopoulou A, Benetou V, Papadimitriou A, Pala V, Palli D, Panico S, Tumino R, Vineis P, Boshuizen HC, Ocke MC, Peeters PH, Lund E, Gonzalez CA, Larrañaga N, Martinez-Garcia C, Mendez M, Navarro C, Quirós JR, Tormo M-J, Hallmans G, Ye W, Bingham SA, Khaw K-T, Allen N, Key TJ, Jenab M, Norat T, Ferrari P, Riboli E: Anthropometry, Physical Activity, and the Risk of Pancreatic Cancer in the European Prospective Investigation into Cancer and Nutrition. Cancer Epidemiology Biomarkers & Prevention 2006;15:879-885
27. Dai Z, Xu YC, Niu L: Obesity and colorectal cancer risk: A meta-analysis of cohort studies. World Journal of Gastroenterology 2007;13:4199-4206
28. Luo J, Margolis KL, Adami HO, Lopez AM, Lessin L, Ye W: Body size, weight cycling, and risk of renal cell carcinoma among postmenopausal women: The Women's Health Initiative (United States). American Journal of Epidemiology 2007;166:752-759
29. Forte V, Pandey A, Abdelmessih R, Forte G, Whaley-Connell A, Sowers JR, McFarlane SI: Obesity, Diabetes, the Cardiorenal Syndrome, and Risk for Cancer. Cardiorenal Medicine 2012;2:143-162
30. Firth SM, Baxter RC: Cellular Actions of the Insulin-Like Growth Factor Binding Proteins. Endocrine Reviews 2002;23:824-854
31. Khandwala HM, McCutcheon IE, Flyvbjerg A, Friend KE: The Effects of Insulin-Like Growth Factors on Tumorigenesis and Neoplastic Growth. Endocrine Reviews 2000;21:215-244

32. Natarajan R, Gonzales N, Xu L, Nadler JL: Vascular smooth muscle cells exhibit increased growth in response to elevated glucose. Biochemical and Biophysical Research Communications 1992;187:552-560
33. Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE: Increased cell division as a cause of human cancer. Cancer Research 1990;50:7415-7421
34. Polet F, Feron O: Endothelial cell metabolism and tumour angiogenesis: Glucose and glutamine as essential fuels and lactate as the driving force. Journal of Internal Medicine 2013;273:156-165
35. Mercer JR, Cheng KK, Figg N, Gorenne I, Mahmoudi M, Griffin J, Vidal-Puig A, Logan A, Murphy MP, Bennett M: DNA damage links mitochondrial dysfunction to atherosclerosis and the metabolic syndrome. Circulation Research 2010;107:1021-1031
36. Botto N, Rizza A, Colombo MG, Mazzone AM, Manfredi S, Masetti S, Clerico A, Biagini A, Andreassi MG: Evidence for DNA damage in patients with coronary artery disease. Mutation Research - Genetic Toxicology and Environmental Mutagenesis 2001;493:23-30
37. Lamprecht SA, Lipkin M: Chemoprevention of colon cancer by calcium, vitamin D and folate: Molecular mechanisms. Nature Reviews Cancer 2003;3:601-614
38. Fenech MF, Dreosti IE, Rinaldi JR: Folate, vitamin B12, homocysteine status and chromosome damage rate in lymphocytes of older men. Carcinogenesis 1997;18:1329-1336
39. Titenko-Holland N, Jacob RA, Shang N, Balaraman A, Smith MT: Micronuclei in lymphocytes and exfoliated buccal cells of postmenopausal women with dietary changes in folate. Mutation Research - Genetic Toxicology and Environmental Mutagenesis 1998;417:101-114
40. Stich HF, Stich W, Rosin MP, Vallejera MO: Use of the micronucleus test to monitor the effect of vitamin A, beta-carotene and canthaxanthin on the buccal mucosa of betel nut/tobacco chewers. International Journal of Cancer 1984;34:745-750

Table 1 - Baseline characteristics of non-diabetic and diabetic individuals

	Non-Diabetic	Diabetic	*P* [a]
N (male/female)	21 (6/15)	76 (34/42)	
Age (years)	62.7±6.30	65.1±7.50	0.180
BMI (kg/m^2)	28.4±3.84	33.6±6.26	0.001
Waist circumference (cm)	96.8±10.0	112.6±15.0	0.000
Fasting blood glucose (mmol/l)	5.63±0.50	8.52±2.20	0.000
HbA1c (%)	5.79±0.26	7.55±0.89	0.000
HbA1c (mmol/mol)	40.0±2.80	59.0±9.70	0.000
Insulin (pmol/l)	58.0±38.6	137±153	0.003
HOMA-insulin resistance	1.97±1.34	7.55±9.42	0.000
MN cells (‰)	0.28±0.29	0.58±0.30	0.000
Total MN (‰)	0.32±0.35	0.71±0.38	0.000
BNC (‰)	22.4±14.3	21.4±13.0	0.885
BE (‰)	0.58±0.94	0.72±0.74	0.089
KR + CC (‰)	25.5±20.7	23.4±16.9	0.944
KL (‰)	56.1±74.5	58.1±69.0	0.930
P (‰)	0.66±0.50	1.10±1.32	0.326
Ex-smokers (n)	8	41	0.226
Years of smoking in the past	7.57±11.9	14.7±16.9	0.092
Quit smoking (years)	5.95±9.52	9.39±13.0	0.245

Data are presented as means ± standard deviations. BMCyt values are per 1000 differentiated cells.

[a] calculated by independent samples t-test (age, BMI, waist circumference, fasting blood glucose, HbA1c, basal cells, yeas of smoking in the past), Mann-Whitney U-test (insulin, HOMA-insulin resistance, MN cells, total MN, BNC, BE, KR + CC, KL, P, quite smoking) and Chi-square test (ex-smoker)

Table 2 - Effect of waist circumference, glucose metabolism and CVD-risk on MN frequency at baseline

	Tertiles	MN frequency (‰)	*P* [a]
Waist circumference (cm)	≤ 102	0.36±0.26	-
	102.1 - 114.7	0.55±0.32	0.066
	≥ 114.8	0.65±0.32	0.004
Fasting plasma glucose (mol/l)	≤ 6.38	0.39±0.30	-
	6.39 - 8.60	0.56±0.37	0.110
	≥ 8.61	0.61±0.24	0.026
HbA1c (%) (mmol/mol)	≤ 6.6 (≤ 49)	0.43±0.35	-
	6.7 - 7.6 (50 - 60)	0.55±0.34	0.074
	≥ 7.7 (≥ 61)	0.61±0.21	0.016
Insulin (pmol/l)	≤ 44.17	0.49±0.35	-
	44.18 - 109.34	0.45±0.27	1.000
	≥ 109.35	0.61±0.33	0.092
HOMA-insulin resistance	≤ 2.03	0.48±0.34	-
	2.04 - 5.44	0.45±0.29	1.000
	≥ 5.45	0.62±0.31	0.072
Adiponectin (µg/ml)	≤ 3.58	0.56±0.30	-
	3.59 - 5.88	0.53±0.33	0.854
	≥ 5.89	0.48±0.33	0.102
CVD-risk	≤ 13.70	0.45±0.29	-
	13.71 - 25.30	0.49±0.37	1.000
	≥ 25.31	0.63±0.26	0.026

[a] refers to comparison with the lowest tertile (Mann-Whitney-U-Test and correction for multiple comparisons)

Table 3 - Characteristics of participants at baseline

	Information	Intervention	*P* [a]
Number (diabetic/non-diabetic)	31 (22/9)	66 (54/12)	
Age (years)	61.7±6.66	65.9±7.19	0.006
BMI (kg/m^2)	32.2±7.03	32.5±5.79	0.820
Waist circumference (cm)	108.5±17.6	109.4±14.5	0.795
Fasting plasma glucose (mmol/l)	7.41±2.07	8.13±2.37	0.150
HbA1c (%)	7.01±1.05	7.26±1.08	0.295
HbA1c (mmol/mol)	53.0±11.5	56.0±11.8	0.295
HOMA-insulin resistance	5.10±3.84	7.09±10.1	0.959
MN cells (‰)	0.44±0.31	0.56±0.32	0.323
Total MN (‰)	0.50±0.37	0.69±0.41	0.106
BNC (‰)	21.7±12.7	21.6±13.6	1.000
BE (‰)	0.48±0.55	0.78±0.86	0.065
KR + CC (‰)	25.0±19.1	23.3±17.1	0.816
KL (‰)	60.7±76.2	56.2±67.2	0.997
P (‰)	0.67±0.84	1.16±1.32	0.014

Data are presented as mean ± SD.

[a] calculated by unpaired t-test (age, BMI, waist circumference, fasting plasma glucose, HbA1c, basal cells) and Mann-Whitney-U-Test (HOMA-insulin resistance, MN cells, total MN, BNC, BE, KR+CC, KL, P)

Table 4 - Changes in genome damage rate in participants of the information and intervention group

	Information			Intervention		
	baseline	week 8	*P* [a]	baseline	week 8	*P* [a]
MN cells (‰)			0.816			0.331
Diabetic	0.53±0.29	0.52±0.36		0.61±0.30	0.51±0.29	
Non-Diabetic	0.22±0.26	0.27±0.26		0.32±0.31	0.40±0.36	
Total MN (‰)			1.000			0.187
Diabetic	0.60±0.34	0.58±0.43		0.76±0.39	0.63±0.39	
Non-Diabetic	0.27±0.35	0.28±0.36		0.36±0.36	0.47±0.45	
BNC (‰)			0.001			0.000
Diabetic	21.3±13.90	15.7±7.42		21.5±12.78	15.0±7.50	
Non-Diabetic	22.8±9.81	13.6±8.45		22.0±17.31	13.3±6.26	
BE (‰)			0.200			0.493
Diabetic	0.62±0.56	0.49±1.29		0.76±0.80	0.71±0.82	
Non-Diabetic	0.16±0.33	0.45±0.36		0.90±1.13	0.62±0.64	
KR +CC (‰)			0.038			0.005
Diabetic	24.3±19.4	15.4±11.1		23.0±15.9	18.6±15.9	
Non-Diabetic	26.7±19.2	23.8±22.0		24.5±22.5	21.3±15.5	
KL (‰)			0.597			0.159
Diabetic	63.3±77.9	60.0±76.9		56.0±65.7	49.3±54.7	
Non-Diabetic	54.3±76.1	41.5±52.7		57.4±76.7	43.9±74.8	
PC (‰)			0.554			0.001
Diabetic	0.74±0.94	0.84±0.88		1.24±1.43	0.59±0.59	
Non-Diabetic	0.49±0.51	0.42±0.50		0.80±0.47	0.57±0.40	

Data are presented as mean ± SD; BMCyt values are per 1000 differentiated cells

[a] *P*-values refer to difference between baseline and week 8 in the information and intervention group (diabetic and non-diabetic individuals together) and were calculated with Wilcoxon-test