From the Department of Biosciences and Nutrition
Karolinska Institutet, Stockholm, Sweden

Oxidative DNA damage and other risk factors, in relation to lifestyle in diabetes type II and metabolic syndrome patients

Rikard Åsgård

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To Eva

Gör alltid det rätta! Det gläder somliga människor och förvånar resten.

Mark Twain

Abstract

Lifestyle factors are important in prevention and treatment of lifestyle related diseases as obesity, type II diabetes and cardiovascular diseases. Patients with these diseases or in the risk zone of obtaining them are classified as having the metabolic syndrome. Diet, physical activity and stress management are considered as important factors for preventing or treating their development. In the development and progress of underlying mechanisms in these diseases, oxidative stress and inflammation are considered as important factors since these patient groups have increased levels of oxidative stress and inflammation compared to healthy subjects.

The aim of this thesis was to study the influence of dietary factors and physiological risk factors on oxidative stress and inflammation in patients with diabetes type II or metabolic syndrome. This has been investigated in two clinical studies, which have resulted in the two papers in this thesis.

In Paper I, patients with metabolic syndrome were part of a residential intervention program for 17 days, with a 5-day follow up. Six months after the start of the program the patients had significantly decreased levels of oxidative stress on DNA. During the same period the patients also had significantly decreased levels of the physiological risk factors weight, BMI, waist- and hip circumferences and body fat, and increased levels of HDL cholesterol. Initial levels and quality of cholesterol were the only factors found to have a correlation to the decrease in oxidative stress. Since the changes probably were lifestyle related, a prolonged lifestyle modification for the patients might help them to maintain the positive results from the intervention.

In Paper II patients with diabetes type II were studied and their dietary intake and relevant clinical risk variables were measured. Correlations between diet and plasma antioxidants with oxidative stress and inflammation were analysed. Plasma α-carotene and β-carotene were found to be good biomarkers for fruit and vegetable intake. Further more, fruit and vegetable intake had a negative correlation with DNA oxidation and lipid peroxidation. Dietary vitamin C had a negative correlation with lipid peroxidation and plasma ascorbate with DNA oxidation. The plasma carotenoids were negatively correlated with inflammation.

In conclusion, a residential intervention program with the purpose to change diet, physical activity level and to cope with stress can result in health benefits for metabolic syndrome patients. Furthermore, antioxidants in fruit and vegetables can be an important lifestyle factor, since it seems to have a positive impact on DNA oxidation, lipid peroxidation and inflammation in diabetes type II patients. Fruit and vegetable intake and ascorbate might affect oxidative stress and carotenoids might affect inflammation, this supporting the recommendations of a high fruit and vegetable intake in this patient group.

List of publications

I. **Åsgård R**, Hedman M, Sjöström M and Möller L.

Long term reduction of DNA oxidation after a life style intervention.

Submitted.

II. **Åsgård R**, Rytter E, Basu S, Abramsson-Zetterberg L, Möller L and

Vessby B.

High intake of fruit and vegetables is related to low oxidative

stress and inflammation in a group of patients with type 2 diabetes.

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Populärvetenskaplig sammanfattning

(Popular science summary)

Övervikt och diabetes

Övervikt och fetma har blivit en epidemi världen över. Typ 2 diabetes är en av följdsjukdomarna. Överviktiga patienter som ligger i riskzonen för att utveckla diabetes eller hjärt-kärlsjukdom brukar diagnosticeras metabola syndromet. De mest typiska symptomen är bukfetma och insulinresistens, som leder till högt blodtryck, blodfettsrubbningar och höga nivåer av insulin och socker i blodet. Detta ökar risken att utveckla typ 2 diabetes och hjärt-kärlsjukdom.

Livsstilen kan fungera förebyggande och behandlande

Genom att förändra livsstilen kan många patienter med metabola syndromet gå ner i vikt och även förebygga och behandla diabetes och hjärt-kärlsjukdom. Kost och fysisk aktivitet är viktiga faktorer att förändra för denna patientgrupp. Även anti-stress program och socialt stöd för att genomföra och bibehålla förändringarna är viktigt, liksom att sluta röka och dra ner på alkoholkonsumtionen.

Syfte

Avhandlingens huvudfrågeställning var att undersöka betydelsen av livsstilsförändringar för patienter med metabola syndromet och/eller typ 2 diabetes, samt att identifiera kostfaktorer som bidrar till en minskad risk för dessa patientgrupper. Oxidativ stress på DNA har studerats som en biomarkör för förändrad sjukdomsrisk hos denna patientgrupp. Även andra relevanta riskfaktorer som fettoxidation och inflammation har studerats. Påverkan av fysiologiska faktorer som t.ex. blodsocker och midjemått på dessa biomarkörer har undersökts. Vidare har intag av frukt, grönsaker och antioxidanter studerats som viktiga livsstilsfaktorer, samt om dessa påverkar oxidativ stress och inflammation hos denna patientgrupp.

Resultat

En tydlig minskning av oxidativ stress på DNA har visats hos en patientgrupp med metabola syndromet efter ett livsstilsförändringsprogram på ett hälsohem, sex månader efter start. Samtidigt förbättrades HDL-kolesterol, vikt, midjemått och kroppsfett, samtliga riskfaktorer för denna patientgrupp. Den enda av de undersökta faktorerna som hade en tydlig relation till förändring av oxidativ stress på DNA var dock kolesterol.

Ett ökat intag av frukt, grönsaker och antioxidanter visade sig bidra till minskad oxidativ stress och inflammation hos typ 2 diabetes patienter. C-vitaminrika frukter och grönsaker verkar påverka oxidativ stress positivt, medan frukt och grönsaker rika på karotener (vitaminer som ingår i A-vitaminer) verkar ha en positiv effekt på patienternas inflammationsnivåer. Antioxidanterna alfa- och beta-karoten i blodplasma visade sig vara bra biomarkörer för frukt- och grönsaksintag.

Slutsatser

Att ett livsstilsförändringsprogram kan skydda DNA från oxidativ belastning hos patienter med metabola syndromet har inte undersökts eller rapporterats tidigare. Resultaten i avhandlingen föreslår att oxidativ stress på DNA som biomarkör kan användas vid utvärdering av andra liknande livsstilsförändringsprogram. Nivåer av och förhållanden mellan LDL- och HDL-kolesterol kan vara en faktor som förklarar varför de flesta patienter minskade oxidativ stress på DNA av programmet, medan några inte uppnådde någon förbättring.

Vikt, midjemått, kroppsfett och HDL-kolesterol är viktiga riskfaktorer för denna patientgrupp, som också förbättrades av livsstilsförändringsprogrammet.

Att intaget av frukt, grönsaker och antioxidanter positivt påverkar nivåer av oxidativ stress och inflammation hos typ 2 diabetiker visar att dessa kostfaktorer bör beaktas vid förebyggande och behandling av typ 2 diabetes och metabola syndromet.

Intag samt nivåer i blodet av C-vitamin verkar ha störst inverkan på nivåer av oxidativ stress, medan nivåer av karotener i blodet verkar ha störst inverkan på inflammationsnivåer, hos typ 2 diabetiker. Vi föreslår därför företrädesvis att frukt och grönsaker som innehåller mycket C-vitamin (citrusfrukter, bär, paprika, vitkål m.fl.) samt frukt och grönsaker som innehåller mycket karotener (mango, morot, tomat, paprika m.fl.) väljs i första hand av typ 2 diabetiker. Våra resultat visar en positiv effekt av intag av antioxidanter från kosten och vi rekommenderar därför en ökning av denna typ av kost. Vi kan naturligtvis inte utesluta att även kosttillskott med dessa antioxidanter kan ha en positiv effekt, men vidmakthåller att ett intag via kosten antagligen blir mer balanserat och därför har en bättre effekt på längre sikt.

Blodplasmavärden av antioxidanterna alfa- och beta-karoten är bra biomarkörer för frukt- och grönsaksintag. Dessa föreslås kunna användas i alla studier där man har möjlighet att mäta dessa plasmaantioxidanter och vill uppskatta frukt- och grönsaksintaget. Förslagsvis så kan dessa mätningar kombineras med någon form av kostregistrering (t.ex. mat-dagbok), för att få en bättre bild av intaget.

Slutligen så vill jag trycka på, utifrån våra resultat, samt många av de studier vi refererat till, att hälsofördelar hos patienter med övervikt, metabola syndromet eller diabetes kan uppnås genom livsstilsförändringar, framförallt en förbättring av kosten, en regelbunden fysisk aktivitet samt att undvika stress. Jag föreslår därför sådana förändringar som ett viktigt komplement vid alla förebyggande och sjukdomsbehandlande åtgärder för dessa patientgrupper.

List of abbreviations

AHA	American Heart Association
BMI	Body mass index (kg/cm^2)
BSA	Bovine serum albumin
CHD	Coronary heart disease
CPT-Vacutainer	Cell preparation tube with sodium heparin
CRP	C-reactive protein (inflammatory biomarker)
CVD	Cardiovascular disease
DH	Diet history
Diabetes type I	Insulin dependent diabetes mellitus (IDDM)
Diabetes type II	Non-insulin dependent diabetes mellitus (NIDDM)
DLW	Double-labelled water
DP	Double portions
E%	Part of the energy intake in per cent
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FFQ	Food-frequency questionnaire
FPG	Formamido pyrimidine glycosylase (DNA repair enzyme)
FPLC	Fast performance liquid chromatography
HDL cholesterol	High density lipid cholesterol
HPLC	High performance liquid chromatography
IDF	International Diabetes Foundation
IHD	Ischemic heart disease
IL-6	Interleukin-6 (inflammatory biomarker)
LDL cholesterol	Low density lipid cholesterol
MDA	Malondialdehyde (lipid peroxidation biomarker)
NHLBI	National Heart Lung and Blood Institute
ROS	Reactive oxygen species
SFA	Saturated fatty acids
TRIS	Tris (nonylphenol) phosfit
WF	Weighed food
8-oxodG	8.oxo-deoxyguanine (DNA-oxidation biomarker)

1. Introduction

The lifestyle has been in focus when discussing strategies for prevention and treatment of certain diseases. These diseases are often called "lifestyle related diseases". Obesity has become an epidemic all over the world, and diabetes type II and cardiovascular diseases can be consequences of a long-term obesity. Obese patients who are in the risk-zone to develop diabetes type II, or cardiovascular diseases, are classified as having metabolic syndrome. Since the lifestyle is important for prevention and treatment in this patient group, it is important to study the impact of these lifestyle factors and also to understand the underlying mechanisms. Especially dietary factors and physical activity have been in focus, as factors to improve health status in these patients.

1.1 Metabolic syndrome and related diseases

Metabolic syndrome

Central obesity (high waist circumference) has been suggested to be an important factor for the diagnosis of metabolic syndrome [1-3]. Central obesity and at least two of the other criteria in Table 1 are required to fulfill the International Diabetes Foundation (IDF) criteria for metabolic syndrome [2]. This is based on the belief that central obesity is an early phase in the etiologic cascade leading to fully developed metabolic syndrome. In contrast The American Heart association (AHA) and the National Heart Lung and Blood Institute (NHLBI) states that three of the criteria in Table 1 have to be fulfilled for the patient to have metabolic syndrome, but central obesity does not have to be one of them [4]. The most typical symptoms for the metabolic syndrome are central obesity with a high waist circumference and insulin resistance, which will lead to high blood pressure, hyperlipidaemia and high levels of insulin and glucose in blood. This will increase the risk of developing diabetes type II or cardiovascular diseases. Additional clinical measures, which have been reported to be associated with metabolic syndrome, are abnormal body fat distribution, atherogenic dyslipidemia, dysglycemia, insulin resistance, vascular dysregulation, proinflammatory state, prothrombotic state and hormonal factors [4]. In the future these factors might be part of the inclusion criteria.

Table 1. At least three of the criteria should be fulfilled for the patients to be classified as a metabolic syndrome patient.

Criteria	Comments
Central Obesity (High waist circumference)	≥94 cm in men, ≥80 cm in women[a] ≥102 cm in men, ≥88 cm in women[b]
Raised triglycerides	≥150 mg/100 ml (1.7 mmol/l) or on specific treatment for this lipid abnormality
Reduced HDL-cholesterol	<40 mg/100 ml (1.03 mmol/l) in men <50 mg/100 ml (1.29 mmol/l) in women or on specific treatment for this abnormality
Raised blood pressure	Systolic: ≥130 mmHg or Diastolic: ≥85 mmHg Or treatment of previously diagnosed hypertension
Raised fasting glucose	≥100 mg/100 ml (5.6 mmol/l) or previously diagnosed diabetes type II or on drug treatment for raised fasting glucose

[a]IDF-criteria [2], [b]AHA/NHLBI-criteria [4].

Obesity

The frequency of overweight persons is increasing all over the world and overweight is coupled to increased risk for disease. The European guidelines for overweight is a body mass index (BMI) of >25 kg/m^2. An increased overweight together with an increased insulin resistance might increase the risk of cardiovascular diseases (CVD) [5, 6]. An increased BMI, among overweight individuals seems to be associated with an increased oxidative stress on DNA in young healthy women [7], and also a raised risk of CVD [8]. Obesity is the diagnosis for people with a BMI ≥30 kg/m^2 [6], and is considered to be an epidemic. Obesity together with insulin resistance increase the risk for CVD [5, 6]. In Table 2, the guidelines for obesity-related risk factors are presented. It has been shown that even "hypertriglyceridemic waist" (>90 cm in men and >85 cm in women), is a significant marker of CVD manifestations occurring at an earlier age in those with glucose intolerance or diabetes type II [9].

Table 2. Guideline with obesity-related risk factor and limits for increased complication and disease together with references.

Obesity-related risk factors	Risk limits	Comments
Overweight	BMI >25 kg/m^2	Increasing overweight together with increasing insulin resistance increase the risk of CVD [5, 6].
Obesity	BMI >30 kg/m^2	Obesity together with insulin resistance increases the risk for CVD [5, 6]
Waist circumference	≥102 cm for men ≥88 cm for women	Increased risk for diabetes type II and CVD [3, 4]
Waist/hip ratio	>1.0 for men and women	An increased waist/hip ratio is associated with a greater risk of death in older persons [10]
Body fat	>17% for men >25% for women	Together with an increased BMI or increased waist circumference the risk for metabolic syndrome increases [11]

The obesity epidemic is important to deal with in many aspects. Individuals that are obese in the mid-life are at increased risk of heart failure and stroke at higher age and may also be at risk for dementia [3]. Both overweight and obesity constitute major public health problems because of the associated increased risk of CHD, stroke, diabetes type II, hypertension, dislipidemia, musculoskeletal disorders and some cancers [12]. It has been shown that waist circumference has the same impact as BMI to identify differences in insulin sensitivity and multiple CVD risk factors [13]. Therefore, either estimate can be used to identify patients at increased CVD risk. The risk factor body fat percentage correlated positively with some studied metabolic risk factors, but most strongly with insulin resistance [11]. The waist-hip ratio, not BMI, was associated with a greater risk of death [10].

Mainly hereditary factors decide the pre-deposition to gain weight. Genetic pre-deposition for diabetes will together with a weight-gaining lifestyle increase the risk to develop diabetes type II.

Lifestyle factors like diet and physical activity can determine the speed of development and the level of obesity. Studies have shown that a higher energy intake and a lower level of physical activity can increase weight and therefore cause obesity. Obese subjects increase the risk to develop diabetes type II, and also diseases like atherosclerosis, hypertension, heart disease, hypoventilation, stroke, hyperlipidemia, gallstones and osteoarthritis [14].

Diabetes type II

The development of diabetes type II or non-insulin dependent diabetes mellitus (NIDDM) is more dependent on lifestyle and environment than genetic factors when compared to type I diabetes. Diabetes type II is a disease where the patients have an elevated risk for oxidative stress and inflammation [15, 16], and a low capacity in the antioxidant protection [17]. In diabetes type II an increased reactive oxygen species (ROS) generation in plasma, and a marked reduction in antioxidant defence that result in oxidative stress in this patient group, coupled to the increased level of blood glucose, can together with the metabolic imbalance be of importance for development of the common secondary complications (Table 3) in the disease [18, 19].

Table 3. Common secondary diseases and symptoms in diabetes type II patients.

Secondary disease or symptom	Comments
Diabetic ocular complications (eyes)	Retinopathy can lead to blindness
Macroangiopathy[22]	Gangrene of the leg, CVD events
Diabetic nephropathy (kidneys)	Linked to diabetic microangiopathy
Atherosclerosis (arteries)	Appears in most diabetics after a few years
Hyperlipidaemia (blood lipids)	> 30 % of patients
Diabetic neuropathy (nerves)	Can lead to bowel and bladder dysfunction

The data in the table is based on reference [14].

It also appears that an increased oxidative stress in diabetes is due mainly to hyperglycemia, which results in stimulation of the polyol-pathway, formation of advanced glycosylation end products, and subsequent formation of ROS [20]. Clinical studies have suggested nutrients

containing antioxidants to be beneficial in reducing oxidative stress in diabetes type II patients [19]. A high intake of fruit and vegetables, rich in antioxidants, is therefore recommended for this patient group [21].

Life style factors, i.e. dietary intake, physical inactivity, and obesity may act as initiation or progression factors for diabetes type II. Therefore, changes in lifestyle would have the potential to prevent or postpone the development of type II diabetes in subjects at high risk. Several well-controlled randomized studies have shown the beneficial impact of a combined dietary and physical activity intervention program on glucose tolerance, insulin resistance, and diabetes development in populations at risk for developing type II diabetes [23]. It has been stated that a combined dietary and physical activity intervention program might prevent one diabetes case per seven participants in such a program over a period of three years [24], or one case per 22 participants over a one-year period [22]. European recommendations for treatment and prevention of diabetes mellitus have been suggested within the Diabetes and Nutrition Study Group (DNSG). A daily consumption of a range of fruit and vegetables to achieve the necessary vitamins and antioxidants and a regular physical activity as well as restriction of energy, fat and sugar intake to avoid increased overweight, have been recommended by the DNSG [21].

Cardiovascular disease

Cardiovascular disease (CVD) is a common denominator for a lot of symptoms and diseases that involve the heart and/or blood vessels. Technically the term refers to any disease that affects the cardiovascular system, but it often refers to those related to atherosclerosis (arterial disease). The risk to develop atherosclerosis increases with age, BMI, blood lipids, hypertension, bad dietary habits, physical inactivity, male gender, cigarette smoking, diabetes and genetic factors [14]. Examples of cardiovascular diseases are; atherosclerosis, angina pectoris, myocardial infarction, ischemic heart disease, stroke, hypertensive heart disease and cardiomyopathy. Common risk factors for cardiovascular diseases are increases in age, weight, cholesterol, inactivity, smoking and also a bad diet and other factors as listed in Table 4 [14].

Table 4. Cardiovascular risk factors

Cardiovascular risk factors	Comments
Age	Other risk factors for CVD than age, increase with age
Absence of nutritional antioxidants	Such as polyphenol antioxidants.
Diabetes mellitus	Both type I and type II.
Hypercholesterolemia	Elevated cholesterol levels and abnormal cholesterol subtypes.
Tobacco smoking	Increase risk for obesity, atherosclerosis and oxidative stress
Higher fibrinogen blood concentrations	Also elevated levels of dimethylarginine amino acid
Elevated homocystein	Even in the upper part of the higher normal interval
High blood pressure	Because of the pressure on heart and arteries
High levels of environmental noise	A correlation with increased CVD risk
Obesity	Especially male-type obesity, presumably by inducing inflammatory and pro-coagulant state.
Genetic factors	Family history of CVD, obesity or diabetes
Physical inactivity	Sedentary lifestyle
Depression	Correlates with obesity and physical inactivity

The table is based on reference [14].

Diabetes type II patients, who had a low-carbohydrate, high-fat and low fiber diet, had a good effect on the blood sugar control but this diet might increase the risk for CVD [25]. Coronary heart disease (CHD) and ischemic heart disease (IHD) are two names of the same disease, with an accumulation of atheromatous plaques within the walls of the arteries that supply the myocardium with blood. This will create a reduction in coronary blood flow. A person can be without symptoms for a long time, when a "sudden" heart attack finally comes [14]. Also diabetes and the duration of diabetes have been shown to be risk factors for fatal CHD [26].

1.2 Lifestyle factors

Lifestyle

Some diseases are considered to be lifestyle related. It has been suggested that one can prevent these diseases and also achieve an improved health and prevent secondary symptoms from the diseases, by lifestyle modifications. Obesity, metabolic syndrome, diabetes type II, cardiovascular disease, rheumatoid arthritis, osteoporosis and stress-syndrome are examples of lifestyle related diseases. Changes in diet and physical activity are considered the most possible individual changes to affect these diseases. Also drug restrictions (tobacco-smoking, alcohol, narcotics), environmental changes and stress management can be considered important changes for these diseases.

There have been few Swedish studies of subjects participating in lifestyle intervention programs at health resorts. In one study, individuals with three or more of the traditional risk factors for CVD (Table 4) participated in a four-week residential program. There were reductions of weight and blood pressure as a consequence of the program and the reductions were significant also after one year. Five years after the program, the weight and blood pressure were still lower compared to the initial values [27]. Another lifestyle modification program of four weeks residential stay and regular contact with a nurse up to one year after, for patients with CVD, was evaluated. Blood lipids, exercise capacity, body mass, anxiety, depression, and self-reported quality of life, were improved and lower rates of all coronary events were the results of the intervention. The best results were achieved when the intervention program was combined with stress management [28, 29].

Lifestyle modification appears effective in delaying or preventing the development of the metabolic syndrome [30], especially physical activity, non-smoking, low carbohydrate intake and moderate alcohol consumption, in subjects with a BMI<30 [31]. Further more, for type II diabetes, social support and resources in the social environment are mediators of lifestyle intervention effects [32]. Lifestyle determinants of cardiovascular risks, like diet, physical activity and smoking, may be useful in preventive medicine as a precocious diagnosis to identify healthy subjects who are at risk for free-radical-mediated diseases [33].

Dietary factors

A national Swedish dietary survey was performed 1997-1998 by the Swedish food administration [34]. The studies showed that the intake of fruit and vegetables, fibres, folate (women), selenium, iron (women), total carbohydrates and fish were too low. The intake of fat, especially saturated fat, mono and disaccharides and food with a high content of sugar and/or energy were too high in the Swedish population when compared to the Nordic nutrition recommendations (NNR) [35]. The NNR are meant to prevent the population from lifestyle related diseases like cardiovascular diseases, obesity, diabetes, some cancers and osteoporosis. The high fat content of the diet in the Nordic countries may have contributed to the high prevalence of CVD, certain types of cancer, obesity and gallstones.

A strong association between the intake of saturated fat, serum cholesterol concentration (LDL-cholesterol) and the incidence of CHD has been shown in cross-cultural studies [35]. Sources for saturated fatty acids and the cholesterol-raising fatty acids are generally found in the same foods, i.e. meat and offals, eggs and diary products. In the Nordic countries no upper limit for cholesterol intake is recommended because the recommended higher intake of fruit and vegetables and lower intake of meat and fats are considered to be better for this purpose than an upper limit.

Studies have shown that the risk of atherosclerosis can remain relatively low, even though the total fat intake varies between 10 and 40 energy per cent (E%), as long as the saturated fatty acids (SFA) do not exceed 10 E%. Replacing saturated fat with unsaturated fat has been shown to be more effective in lowering the risk of CHD than reducing total fat consumption [36]. On the other hand, for body weight control in populations with low physical activity and high prevalence of obesity, restriction of total fat intake can be beneficial [37]. By reducing total fat intake, or by modifying dietary fatty acid composition in order to reduce the proportion of SFA and increase that of unsaturated fatty acids, the risk to develop CHD is reduced. In studies exceeding six months in duration, reduction or modification of dietary fat intake reduced cardiovascular events by 16% [35]. Also, studies have been performed in the Nordic countries showing that replacing SFA with unsaturated fat can improve insulin sensitivity in healthy subjects when total fat intake is kept below 37 E% [38] and that reduction of total saturated fat together with weight reduction and increased physical activity reduces the risk of diabetes in subjects with glucose intolerance

[22]. The European Diabetes and Nutrition Study Group have given recommendations for diabetes type II patients. An increase of physical activity parallel with a decrease of the energy intake is recommended. Further more, an increased intake of fruit and vegetables is recommended since it has shown to be beneficial for this patient group [21].

Consumption of fruits and vegetables is associated with a lower risk of several chronic diseases including cardiovascular diseases and mortality [39]. A high consumption of fruit and vegetables has a protective effect for cardiovascular diseases [40] and for diabetes type II [21]. The recommended intake of fruit and vegetables in Sweden is 500g/female/day and 700g/male/day respectively [35]. Questionnaire methods, such as food-frequency questionnaires (FFQs) and diet history (DH), are commonly used methods to assess individuals´ diets in case-control and cohort studies. To validate FFQ and DH, 24-hour recalls are mostly used [41]. A Food diary (3, 4 or 7 days) can be used to find out the dietary contents. Validation of food diaries can be done by comparison with weighed food (WF), double portions (DP) and/or double-labelled water (DLW) [42].

Fruit and vegetable intake seems to be a safe and balanced way to achieve the dietary antioxidants needed. Dietary supplementation with antioxidants is another source, but in this case the data from different studies are more contradictory. A supplementation of up to 180 mg β-carotene per day has been used for years without inducing vitamin A toxicity [35], but in a meta-analysis from 2007 of mortality in randomised trials of antioxidant supplementation, β-carotene, vitamin A and vitamin E supplementation was found to have a relation to increased mortality [43]. Supplementation with the carotenoid lutein has provided both null and positive effects on biomarkers for oxidative stress. Associations between lutein and a lower risk of developing cardiovascular diseases as well as some types of cancer have been seen in epidemiological studies [44]. Further more, an increased intake of vitamin C raises plasma levels of vitamin C, which in turn raises serum HDL cholesterol and lowers serum triglyceride levels [45], and also vitamin C has been shown to be protective against stroke and probably against coronary heart disease [46]. In conclusion, dietary intake of antioxidants in the form of fruit and vegetables, seems to be a better source with lower risks compared to supplements.

Physical activity

Moderate physical activity on a regular basis might decrease the risk to develop obesity, diabetes type II, hypertention and CHD [14, 47, 48], and can improve insulin sensitivity [35]. Even light-to-moderate physical activity might decrease the risk for obesity and type II diabetes [49], and physically active persons have only half the risk for CHD compared to physically inactive persons [50]. Physiologically, regular physical activity has positive associations with plasma lipids [51] and insulin sensitivity [36]. In contrast to this, physical inactivity has been shown to increase the risk of obesity and type II diabetes in women [52], and increase vascular superoxide production and atherosclerotic lesion formation in mice [49]. Endurance exercise has been shown to result in DNA damage, as detected by the comet assay [53]. In the NNR 2004, physical activity recommendation was a new and important part [35]. A daily exercise of 30 minutes of moderate or high intensity to achieve health advantages was recommended, especially to affect symptoms present in metabolic syndrome. A daily exercise of 60 minutes can also give a weight reduction and prevent further obesity, cardiovascular diseases and diabetes type II [35, 54].

A lifestyle intervention program at a typical Swedish health resort

The Österåsen health resort in the north of Sweden, owned and run by the public health care, has a lifestyle intervention program for patients with metabolic syndrome and other lifestyle related diseases. The program is described more in detail in Paper I. Most patients at the health resort have been referred from a physician to take part in the intervention program for six months. The aim of the intervention is to change lifestyle mainly in the areas of food intake and physical activity, and teach how to deal with the difficulties to change lifestyle in their ordinary life at home. This can be achieved through information, support from their environment and tools to change habits towards a healthy lifestyle.

The patients start with a basic course of 17 days with full-day programs of activities. When they come home they continue to practice the new healthy lifestyle and come back for 5-day follow-up courses after 3 months and after 6 months. The patients keep in contact with the health resort

during the 6 months intervention. At the health resort there is a mix of conventional medicine (physicians, nurses, physio-therapists and dieticians) and complementary health care.

Figure 1. The patients at the health resort will only be served low sugar, low fat, lacto-vegetarian food. The lunch buffet always has a hot dish together with a large sallad-buffet.

In Figures 1-3 the main three areas in the intervention program; diet, physical activity and group sessions, are presented with images from the health resort. The lacto-vegetarian lunch buffet at the health resort, low in energy, sugar and fat, is tasty and appreciated by the participants (Figure 1). It is known that for these patient groups, a change of diet towards a healthier one can increase health status and improve physiological risk factors [14]. The aim at the health resort is to teach the patients to cook and eat healthy food, but also to maintain the new healthy food habits at home.

Figure 2. The obese patients with metabolic syndrome are trained to increase their physical activities. Water gymnastics is a popular activity that is easy to practice for the patients.

In Figure 2, a typical physical activity at the health resort, water gymnastics, is presented. Another popular activity among these patients is stick-walking. These are activities that even the very obese patients can continue to practice at home. For many of these patients a training program is received from a physio-therapist, and practised daily at the health resort.

Another important part of the intervention program includes group sessions. Practical, theoretical and therapeutic sessions in small groups are used during the program. Cooking lessons, stress management, theme groups, goal settings and cognitive behavioural therapeutic work are examples of the group sessions (Figure 3).

Figure 3. The group sessions include cooking lessons and goal settings worked out in groups. Cognitive behavioural therapy influences all group sessions.

1.3. Oxidative Stress and inflammation

Reactive oxygen species

Reactive oxygen species (ROS) are common denominators for radicals and non-radicals involved in oxidation of biological molecules (Table 5). Free radicals can be defined as any species containing one or more unpaired electrons, and are therefore very reactive and important intermediates in lipid- protein- and DNA oxidation [55]. In addition, non-radicals can be intermediates in these oxidative processes. Neither $O_2^{\bullet-}$ nor H_2O_2 is particularly reactive in aqueous solution, and both $O_2^{\bullet-}$ and H_2O_2 can act as both oxidizing and reducing agents in different systems in aqueous solution. Several biologically important molecules oxidize in the presence of O_2 to yield $O_2^{\bullet-}$. In the body transition metals, like iron or copper, are present, which will increase oxidation. The highly reactive $OH^{\bullet}$ radical can be generated in biologically relevant systems by multiple reactions. Two examples are Fenton reaction and UV-induced homolytic fission of the O-O bond in H_2O_2, which will form $OH^{\bullet}$ [55]. Patients with elevated levels of DNA base oxidation products, in white blood cell DNA in diabetes type II patients, have been shown to have an increased formation of $OH^{\bullet}$ attacking DNA, but no increased formation of reactive nitrogen or chlorine species [56].

Increased free radical formation will greatly accelerate DNA damage. ROS can damage DNA temporarily or permanently, but $O_2^{\bullet}$, NO or H_2O_2, at physiologically relevant levels, do not appear to react with any of the DNA or RNA bases, or with the ribose or deoxyribose sugars, at significant rates. In contrast, as might be expected from the high reactivity of the $OH^{\bullet}$ radical, exposure of DNA to $OH^{\bullet}$ generates a multitude of products, since it attacks sugars, purines and pyrimidines [57]. For example, $OH^{\bullet}$ can add to guanine at positions 4, 5 and 8 in the purine ring. Addition to C-8 produces a C-8 OH-adduct radical that can be reduced to 8-hydroxy-7,8-dihydroguanine (8-oxydG), as shown in Figure 4, oxidized to 8-hydroxyguanine, or undergo opening of the imidazole ring, followed by one-electron reduction and protonation, to give 2,6-diamino-4-hydroxy-5-formamidopyrimidine, usually abbreviated as FAPyG.

There are defence mechanisms against these DNA damaging agents in the form of antioxidants and DNA repair enzymes. The function of a repair system can be used in an assay to measure

DNA damage. E.coli formamido pyrimidine glycosylase (FPG) is one DNA-repair enzyme that is used in the Comet assay to cut out a range of oxidized purines [58], which makes it possible to use the Comet assay to measure oxidative stress on DNA (see Paper I and Paper II).

Table 5. Reactive oxygen species (ROS), radicals and non-radicals.

Radicals	Non-radicals
Superoxide, $O_2^{\bullet-}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Hypochlorous acid[a], HOCl
Peroxyl, $RO_2^{\bullet}$	Ozone, O_3
Alkoxyl, $RO^{\bullet}$	Singlet oxygen, $^1O_2$
Hydroperoxyl, $HO_2^{\bullet}$	Peroxynitrite[b], $ONOO^-$

[a]Can also be called reactive chlorinated species, [b] Can also be called reactive nitrogen species.

Oxidative Damage on DNA

Oxidative stress on DNA is an imbalance between oxidative pressure on DNA and efficiency of the defence system (antioxidant capacity and DNA repair). Appropriate levels of antioxidants and an efficient DNA repair capacity are important protective factors in the process of ageing and the development of cardiovascular diseases and inflammatory diseases [59]. Furthermore, oxidative stress plays an important role in the pathogenesis of several diseases such as diabetes type II and cardiovascular diseases and is associated with life style factors such as low consumption of vegetables, fruit and fish as well as stress, tobacco smoking and alcohol consumption [33]. Co-supplementation with iron and vitamin C had positive effects on the levels of oxidative DNA damage in white blood cells [60]. A common biomarker of DNA-oxidation is 8-oxodG, which is in equilibrium with 8-OH-dG, shown in Figure 4 together with deoxyguanosine (dG). 8-oxodG can serve as an example of a commonly oxidised DNA base and it can be analysed in cells, plasma or urine. In paper II, 8-oxodG has been analysed in human lymphocytes and monocytes with HPLC/EC/UV. There have also been studies to measure 8-oxodG in urine [61], as well as the excretion of 8-oxodG from cells irradiated by ionising irradiation *in vitro* including leukocytes or lymphocytes. The last mentioned two variants were analysed with both HPLC and

ELISA (with antibodies specific for 8-oxodG) [62] and have been useful for more long-term measurements of oxidative stress on DNA.
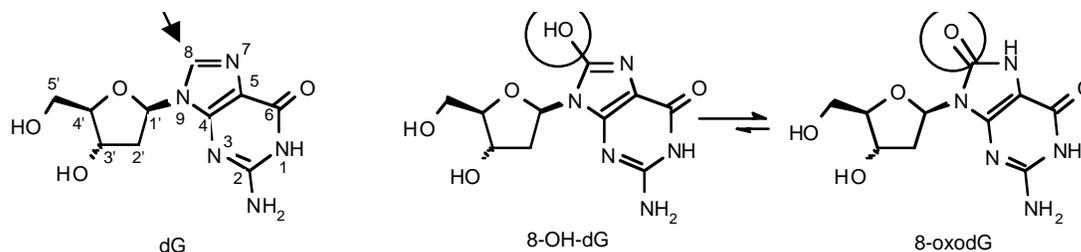

Figure 4. Structures of dG, and oxidized forms of dG (8-OH-dG and 8-oxodG). The arrow shows the oxidation site at the carbon in position eight and the circles show the oxidation products at the same site.

Lipid peroxidation

All lipids in the human organism can be oxidised, but there are several defence mechanisms against the oxidation. Antioxidants can protect the lipids from oxidation and enzymes, like glutathione peroxidases, can convert peroxides to alcohols. Lipid peroxidation has been defined by A.L. Tappel as "the oxidative deterioration of polyunsaturated lipids" [55]. Polyunsaturated fatty acids are those that contain two or more carbon-carbon double bonds (PUFAs). The membrane that surrounds cells and cell organelles contain large amounts of PUFA side chains and the major constituents of biological membranes are lipids, such as lecithin, cholesterol and phospholipids, and proteins. Dietary fats are digested, absorbed and transported inside the organism. The lipoproteins in these processes are targets of oxidative stress. Oxidation of lipids and lipoproteins can be harmful. Peroxidation of low density lipoproteins (LDL) will for example lead to atherosclerosis. Initiation of lipid peroxidation is caused by attack on lipids of any species that can abstract a hydrogen atom from a methylene (-CH2-) group. PUFAs are sensitive to these attacks. Initiation events can lead to formation of multiple molecules of peroxide. Peroxidation of linoleic acid gives two hydroperoxides. Peroxidation of arachidonic acid will give six lipid hydroperoxides as well as cyclic peroxides and other products, including the isoprostanes [55].

To quantify lipid peroxidation a number of assays are available, among them 8-iso-PGF2α, an oxidation product of prostaglandins. Isoprostanes are prostaglandin derivates mainly formed by peroxidation of arachidonic acid catalysed by free radicals. Free or total 8-iso-PGF2α can be

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quantified with different analysis techniques including gas chromatography-mass spectrometry, liquid chromatography, enzyme immunoassays and radioimmunoassay [63].

Antioxidants

Aerobic organisms have evolved antioxidant defences against ROS. Antioxidant defences include enzymes (catalase, peroxidase and superoxide dismutase) that catalytically remove free radicals and other reactive species, proteins that minimize the availability of pro-oxidants such as ions of iron and copper (transferrin, haptoglobins and metallothionein), protective proteins (heat shock proteins) as well as low-molecular weight scavengers of ROS and RNS (glutathione, α-tocopherol, β-carotene and ascorbate). In living organisms, many antioxidants come into action when ROS and RNS are generated [64]. Intake of antioxidants has been proposed to prevent diabetes complications, but this is probably only a part of the solution [65-67].

In vitro, ascorbate (vitamin C) has been shown to have a multiplicity of antioxidant properties, protecting various biomolecules against damage by ROS or RNS [55]. However, ascorbate in high concentrations may increase levels of hydrogen peroxide and therefore increase oxidative stress [55, 68, 69]. Ascorbate *in vitro* scavenges ROS and RNS, protects plasma lipids, membranes and lipoproteins and co-operates with vitamin E through regeneration of α-tocopherol.

Vitamin E (mainly α- and γ-tocopherol) is a scavenger of peroxyl radicals and therefore protects against lipid peroxidation while it also has the ability to protect membranes against ROS [55]. However, vitamin E can sometimes act as a pro-oxidant, for example by reducing Iron or Copper, and is therefore not an obvious antioxidant in the human body. Furthermore, among E-vitamins α-tocopherol and γ-tocopherol can act as antagonists in the body. Low plasma concentration of γ-tocopherol may be caused by a high intake of α-tocopherol, due to the competition between α- and γ-tocopherol for the tocopherol binding proteins, which have high affinity for α-tocopherol [70]. Generally, α-tocopherol is more frequently found in the body while γ-tocopherol is more abundant in diet.

Carotenoids (among A-vitamins) have been suggested to exert antioxidant protection by scavenging DNA-damaging free radicals and modulation of DNA repair mechanisms [71]. Since

both ascorbate and vitamin E have been found to act both as antioxidants and pro-oxidants, the carotenoids might be the most promising dietary antioxidants for the humans.

Role of oxidative stress in metabolic syndrome and diabetes

Diabetes mellitus is characterized by high levels of glucose in the blood. The hyperglycemia initiates the formation of glycated proteins, glucose oxidation and increased fatty acids [65]. This results in oxidative stress in the mitochondria, as well as activation of oxidative and inflammatory signalling pathways, which continue to damage the insulin-producing cells. This will result in retinopathy, nephropathy, atherosclerosis and subsequent coronary artery disease, cerebral vascular disease and peripheral artery disease, and can be related to microangiopathy or endothelial injury [72]. In the prediabetic state, metabolic syndrome, insulin resistance, hypertension, and dislipidemia increase the risk of CVD [73].

Life style factors like high-caloric foods and decreased physical activity resulting in obesity, can lead to insulin resistance. The glucose toxicity can result in abnormal fatty acid metabolism and autoxidation of glyceraldehyde, which generate hydrogen peroxide and α-ketoaldehydes and can lead to chronic oxidative damage [74]. Formation of glycation products and sorbitol is important in the pathogenesis of the diabetes complications, and is also involved in ageing, resulting in DNA strand breaks and reactive dicarbonyls [75]. In diabetic subjects, the oxidative stress can target the β-cell in the pancreas, alter its function and result in an increased insulin resistance, because of lost insulin gene expression in the islet cells. The hyperlipidemia can also result in fatty acid mediated oxidative damage and metabolic disturbances in the β-cell in the pancreas [74]. Further more, acute hyperglycemia seems to provoke oxidative stress, which destroys the natural antioxidant defences found in the plasma [76].

Inflammation

Inflammation relates to oxidative stress and also to metabolic syndrome and diabetes. Oxidative stress generally starts by local events, while inflammation always includes systemic activation of the immune system. In the acute phase inflammation, the immune system is activated because of tissue injury. Interleukin-6 (IL-6), a commonly used biomarker for inflammation, is mainly

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formed by monocytes and macrophages and can start a systemic response to a local inflammatory damage [40]. This can result in production of acute phase proteins in the hepatocytes measured as for example C-reactive protein (CRP), which will be excreted into the circulation and a systemic inflammation occurs. Furthermore, 15-keto-dihydro-PGF2α, a metabolite of an oxidized prostaglandin, could be used as a biomarker for inflammatory response *in vivo* [77].

2. Aim

2.1 General aim

The general aim of this thesis was to investigate the influence of lifestyle factors on oxidative DNA damage and other risk factors, in metabolic syndrome and type 2 diabetes patients. Main focus was to study if a lifestyle intervention program or lifestyle factors such as intake of fruit, vegetables and antioxidants can affect levels of oxidative stress and inflammation in these patient groups.

2.2 Specific aims

• To investigate if a lifestyle intervention program can affect oxidative stress on DNA in metabolic syndrome patients.

• To investigate if the same lifestyle intervention program also can affect other risk factors such as BMI, waist circumference, body fat, blood sugar and blood lipids in metabolic syndrome patients.

• To study correlations of dietary intake and plasma antioxidants with oxidative stress and inflammation in a group of diabetes type II patients.

• To investigate the dietary intake, with a focus on fruit, vegetables and antioxidants, plasma antioxidant levels, as well as levels of oxidative stress and inflammation in a group of type 2 diabetes patients with a stable metabolic control.

3. Materials and methods

3.1 Procedures in the lifestyle intervention study (Paper I)

Study population and lifestyle intervention program

Twenty-six metabolic syndrome patients (52±5 years; 94±18 kg; BMI 32±5 kg/m^2) participated in a life style intervention program for six months. Between the patients, their referring physician and the health resort, a treatment agreement was arranged. Exclusion criteria were drug problems, acute medical needs or other situations that made the patient unsuitable to take part in the program.

The patients were staying for 17 days at the health resort, Österåsen, in the north of Sweden, and were then followed up for a period of five days at the resort after three and six months, respectively, for feed-back, to improve goal settings and to give further instruments to maintain a long-term healthy life style. The aim of the life style intervention program, based on cognitive theories, was to make the patients change their attitude and behaviour, mainly in the areas of food intake and physical activity. The program was complementary to the conventional medical treatment prescribed by the physicians in the primary health care system.

The intervention program included: (1) individual consultations, medical testing and goal setting with physician, nurse, dietician and physiotherapist, (2) lectures and group discussions, (3) theory and practice regarding healthy (mainly vegetarian) food, (4) daily physical exercise with water gymnastics, floor gymnastics, stick-walking, spinning, dancing and out door walking, and (5) relaxation exercises. Lectures and practical sessions were mixed during the course.

The anthropometric measurements and the blood sampling were carried out with fasting patients, wearing light indoor clothes but no shoes, in the morning before start of the intervention and six months after. The levels of the 26 patients after the intervention were compared to their own initial values (controls). Ethical permission (02-366) for the study was attained from The Scientific Ethical Committee of Umeå University, Sweden.

Blood sampling and cell collection

The blood sampling was carried out in the morning with fasting patients. For the separation of mononuclear blood cells for further analyses of DNA damage a sample of 4 ml blood from each patient was collected in CPT Vacutainer cell preparation tubes. The tubes were kept cold and dark and were transported to the laboratory. Separation of mononuclear white blood cells was performed within 4h from blood sampling, by centrifugation of the CPT tubes at 1650 x g for 20 min at 12°C. The plasma was collected and frozen at -80°C. The cells were centrifuged in cold RPMI 1640 medium containing L-glutamine 700 x g for 15 min at 4°C. The medium was removed and the cell pellet from the patient was resuspended in freezing medium, separated in 5 aliquots of 200 µl cell suspension and after that slowly frozen to –80°C and stored until further analysis with the Comet assay. In Paper II the same blood sampling and cell collection protocol was used to prepare human mononuclear blood cells for the Comet assay and for 8-oxodG analyses. The freezing medium was made of heat-inactivated fetal bovine serum containing 10% dimethylsulfoxide. The protocol has, with minimal changes, been presented previously [7].

Oxidative stress on DNA

A historical view on the Comet assay
Single cell gel electrophoresis (the Comet assay), is a well-used toxicological method to measure DNA damage. Östling and Johansson first developed the assay in 1984 [78]. Individual cells (any eukaryotic cells can be used) are mixed with low temperature melting agar and put on a microscope slide. The cells are lysed and the DNA unwinded. Electrophoresis at neutral pH brings the damaged DNA out of the cell. Marked with a DNA-binding staining this damaged DNA forms a tail [79], and the tail consists of single stranded DNA pieces.

  Several modifications of the assay have been performed. Singh et al 1988 lysed the cells at pH 10 with 2.5 M NaCl followed with a treatment with alkali (0.3 M NaOH) and electrophoresis at the high pH>13 [80]. Olive et al 1990 lysed the cells in low alkali (0.03 M NaOH and 1 M NaCl) before electrophoresis in low alkali [81]. In the high alkali variant, alkali labile sites (ALS) will on top of the other single strand breaks also be changed to single strand breaks and measured in

the method [79]. Both the low alkali and the high alkali variant of the method give a very sensitive method to measure DNA-damage.

A variant of the method was introduced with addition of DNA repair enzymes like UV-endonuclease, Endonuclease III or Formamido Pyrimidine Glycosylase (FPG) [82, 83]. These enzymes will, on top of the other DNA damages measured in the method also cut out oxidized DNA bases. FPG is a DNA repair enzyme that mainly cuts out oxidized purines (8-oxodG, 8-oxodN, 8-oxodI, 8-oxodA, fapy-dG, me-fapy-dG, fapy-dA, ROP-3, ROP-3a, dG-ro) and transfers them into extra strand breaks in the Comet assay [58]. Therefore, oxidative DNA damage can be analysed when comparing FPG-treated with untreated samples in the Comet assay. In the EU-project ESCODD including 28 laboratories in Europe, 8-OH-dG with HPLC EC/UV and the high alkali variant of the Comet assay with FPG were used to measure oxidative DNA-damage [84]. This variant has been used in Paper I-II since it will give sensitive and stable analyses of oxidative DNA damage. The important steps in the method are illustrated in Figure 5.

The Comet assay, high alkali variant, with FPG

In this study, the high alkaline Comet assay with Formamido pyrimidine glycosylase (FPG) was used, which is a well established method and also validated in an extensive European collaboration study in the area of biomarkers for measuring oxidative DNA damage [7, 84, 85]. The cells were kept on ice and in darkness during the whole procedure to prevent auto-oxidation. Lymphocytes and monocytes in 200 µl aliquots were thawn gently in a 37°C water bath and kept on ice. One ml of RPMI 1640 medium (+4°C) containing L-glutamine with 10% heat-inactivated fetal bovine serum was added drop-wise, immediately followed by centrifugation at 200 x g for 3 min (4°C). The supernatant was discarded and the cells were washed in PBS and centrifuged at 200 x g for 3 min at +4°C. The cell pellet was mixed in low gelling temperature agarose on 3-well microscope slides.

Thereafter, the cells were lysed in a pH 10 buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris with 1% Triton X-100) for 1 h at +4°C, leaving super coiled DNA embedded in the agarose. Incubation in enzyme buffer with pH 8 (0.1 M KCl, 0.5 mM EDTA, 40 mM Hepes, 0.2 mg/ml bovine serum albumin BSA) for 2x7 min at +4°C, will prepare the DNA for the FPG-treatment. The cells were then treated with Formamido pyrimidine glycosylase (FPG) 500 units/100 µl diluted 1:120 in the enzyme buffer to a final concentration of 4.2 units/µl. Diluted FPG (30

µl/field), or enzyme buffer for controls, were added to round microscope fields in a humidity chamber at 37°C for 30 min. FPG-enzyme cut out oxidised bases in the DNA. Unwinding in 0.3 M NaOH with 1 mM EDTA at pH>13 for 40 min prepared the cells for the electrophoresis, which was performed in a Sub-Cell GT unit for 30 min in the same high alkali solution at 25 V ($\approx$ 0.86 V/cm).
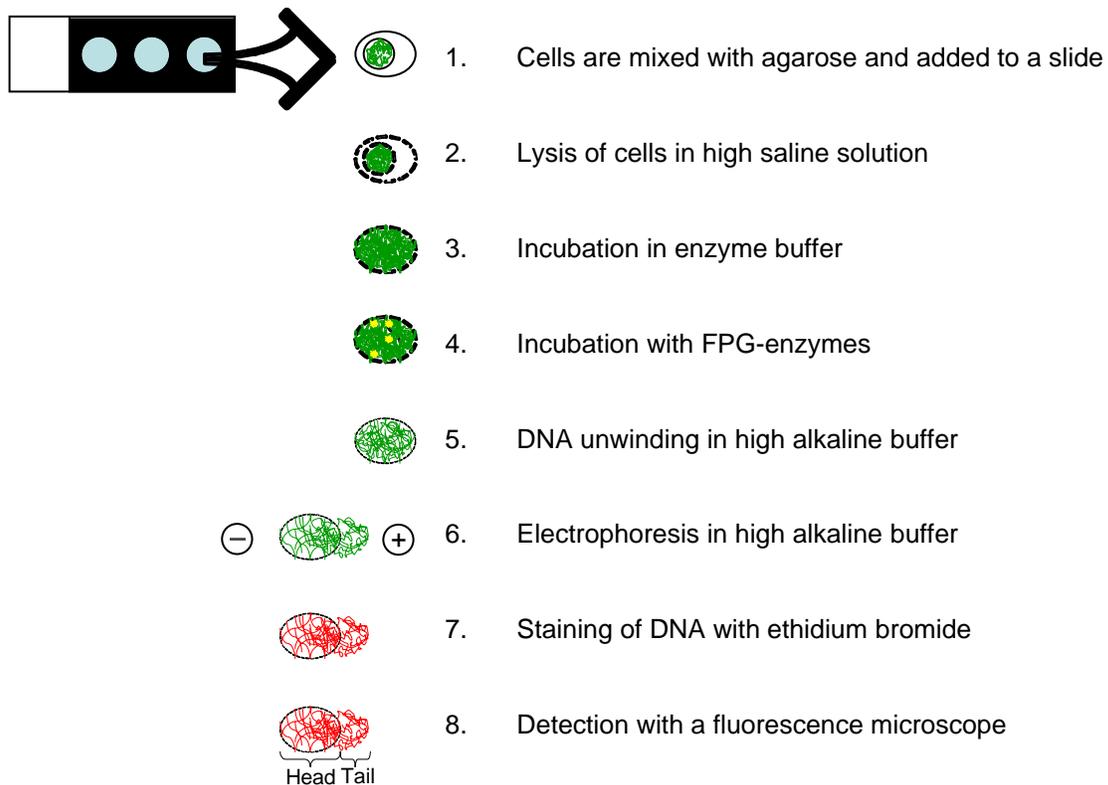

1. Cells are mixed with agarose and added to a slide

2. Lysis of cells in high saline solution

3. Incubation in enzyme buffer

4. Incubation with FPG-enzymes

5. DNA unwinding in high alkaline buffer

6. Electrophoresis in high alkaline buffer

7. Staining of DNA with ethidium bromide

8. Detection with a fluorescence microscope

Head Tail

Figure 5. The high alkaline Comet assay with FPG-enzyme treatment. The mononuclear white blood cells (1) are mixed with agarose and the mixture is added to a microscope slide. The DNA is coloured green (1-6). The lysis (2) will wash everything away except for the DNA (green). The enzyme buffer (3) will prepare the DNA inside the "head" for the electrophoresis. The FPG-enzymes (yellow) will cut out oxidised DNA bases (4). High alkaline buffer (5-6) will unwind the DNA and make strand breaks at alkali labile sites. The electrophoresis (6) will bring the damaged DNA out of the "head" to form a "tail". Staining in ethidium bromide gives the DNA a red colour (7). The fluorescence intensity of the tail, compared to the head, will be detected in a fluorescence microscope (8) and analysed with an image analysis program. The percentage damaged DNA out of all DNA in the cell image will be measured (% Tail). By subtraction of damage level in untreated cells from damage level in FPG-treated cells the level of oxidative DNA damage can be analysed.

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DNA, which is negatively charged, moved as smaller single-stranded fragments towards the anode and formed a tail of damaged DNA. The longer strands of undamaged DNA stayed in the round "comet head". The more damaged DNA, the larger the tail. Neutralization was performed in 0.4 M Tris for 2 x 5 min, after which the samples were washed for 5 min in water, dried over night and then fixed in methanol for 5 min.

Staining of DNA was performed with 10 µg/ml ethidium bromide in Tris-acetate-EDTA (TAE, pH 7.8) for 5 min and thereafter the excess of ethidium bromide was washed away during 5 min in a TAE buffer. The difference in intensity between the "comet tail" and the "comet head" was assessed in a fluorescence microscope with a 20 x lens, and a computerized image analysis program Komet 4.0 was used to calculate the percent damaged DNA. Tail % was used to describe how large percent of the DNA that was damaged. Both cells (n=50) treated with FPG and cells (n=50) without FPG treatment were analysed, for each patient and time point. The oxidative DNA damage levels were calculated by subtraction of untreated control cells from FPG treated cells. The protocol is described in detail elsewhere [84]. A microscope image of a comet with a high level of damaged DNA is shown in Figure 6, to illustrate the method.
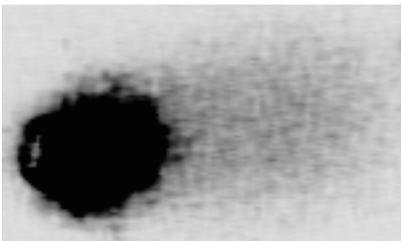

Figure 6. An example of a comet with a high level of damaged DNA, is here illustrated with an image from the microscope. The dark round area to the left is called the "head" and contains the undamaged DNA. The area to the right, full of small dark dots, is called the "tail" and contains the damaged DNA. A larger "tail" means that a larger part of the DNA in the cell is damaged. Cells with no damaged DNA will show no tails in the Comet assay. The DNA-content in the tail is quantified with an image analysis program.

Statistics

Changes in anthropometrics and blood values and correlations between damage versus anthropometrics or blood values at baseline as well as six months in the program were tested by paired t-test. The significance levels were adjusted for mass significance by the Bonferroni-Holms method. The percent damaged DNA in the cells were analyzed with 3 way ANOVA with two levels of damage; not FPG treated (genotoxic damage) and FPG treated (genotoxic damage with oxidative damage), time point (baseline and six months) and patients (n=26), the first two factors fixed and the third random. Tukeys test was used to make pair wise comparisons of genotoxic damage and FPG treated damage over time. The significance level was 5 %. Students´ t-test was used to test differences between upper and lower quartiles [86].

3.2 Procedures in the diabetes type II patient study (Paper II)

Study population and study design

The participants were recruited to take part in an intervention study with antioxidant supplementation. The measured variables at baseline and their correlations are described separately in Paper II.

The subjects underwent assessment and completed a self-administered questionnaire to collect data for inclusion/exclusion. The inclusion criteria were 40-75 years of age, diabetes type II treated with either oral hypoglycaemic medication or diet, HbA1c < 10, BMI < 35 and a stable body weight the last 3 months. Subjects with insulin dependent diabetes and acute inflammatory diseases and diseases of liver, kidney and thyroid gland were excluded. Medication or supplementation that could possibly affect antioxidative, oxidative or inflammatory status was a reason for exclusion. Treatment with non-steroidal anti-inflammatory drugs was a reason for exclusion but low dose treatment (75 mg) with acetyl salicylic acid (ASA) was accepted.

Food supplementation was not allowed 1 month before study start, but 2 subjects who took food supplements until just before study start were included since they fulfilled all other inclusion

criteria. All dietary and plasma antioxidant data in Paper II were analysed without including these subjects. In the correlations with inflammatory biomarkers in Paper II, 2 subjects with CRP>10 were not included in the analyses. Altogether 56 patients participated in the study, but 54 subjects were included in the data of Paper II (28 women and 25 men in the dietary data and 27 women and 24 men regarding the inflammation data). Also the data for folate, B12 and micronuclei only included 36 subjects.

   Paper II can be considered as a cross sectional study of patients with diabetes type II, using baseline data from a randomised double blind, parallel placebo-controlled intervention study. Blood and urine samples were drawn, and body height, weight and waist circumference were carried out with fasting patients in the morning before study start. The measurements were performed as described in Paper II.

Subjects received oral and written instructions to restrain from alcohol intake and heavy physical activity the day before and the same day as the clinical examination.

The study (Paper II) was approved by the Ethical Committee of the Medical Faculty at Uppsala University, Sweden (Dno 02-502). The subjects gave their written consent to participate in the studies.

Dietary intake

53 of the 54 participants completed a 3-day dietary survey one week before entering the study. Subjects were asked to record everything they ingested for two weekdays and one weekend day in the food diary consisting of totally 119 food items. The weekdays recorded were randomly represented among the participants. The food diary used was validated in a 7-day registration [42] and among other studies used in a nationwide dietary survey in Sweden called "Riksmaten" [34]. The 3-day food diary "Menyboken", used in Paper II was precoded to be analysed with computorised software, where macro- and micronutrient, food groups and fruit and vegetable intake were obtained. Further analyses were achieved on a food-group level as for fruit and vegetable intake. Fruit and vegetable intake was categorised, as being the intake of vegetables, root crops, fruit, berries, as well as marmalade, jam, stews and preserves made of fruit, berries or root crops.

Plasma antioxidants and blood analyses

Plasma carotenoids; α-carotene, β-carotene, lycopene and lutein, were detected by HPLC, while plasma ascorbate concentration was analysed by a fluorometric method. The amount of α-tocopherol and γ-tocopherol in serum was analyzed with HPLC, and adjusted for the sum of the cholesterol and the triglyceride concentrations. The analyses of serum folate and vitamin B12 were done at the Clinical Chemistry and Pharmacology Centre for Laboratory Medicine, Uppsala University Hospital, Sweden. Blood glucose concentration was analysed by enzymatic techniques and HbA1c was analysed with fast performance liquid chromatography (FPLC). Plasma insulin was assayed with an enzymatic immunological assay.

Oxidative stress on DNA

Blood sampling, cell collection and the Comet assay analyses
Blood sampling, collection of mononuclear white blood cells, and analyses of DNA oxidation with the Comet assay were performed with the same protocol as in Paper I.

8-oxodG with HPLC EC/UV
The collected mononuclear white blood cells were used for analyses of 8-oxodG. Work-up procedures were performed on ice and as quickly as possible to minimise artefactual oxidation. All aqueous solutions were treated to remove metal ions, and then filtered. After that homogenisation, nuclei preparation and DNA isolation were performed. DNA pellets were dissolved and the DNA was hydrolysed enzymatically. The DNA hydrolysate was transferred into a filter and the filters were centrifuged. The DNA hydrolysates were stored at -80 °C until analysis. HPLC-EC/UV systems together with an autosampler were used with an electrochemical detector for the detection of 8-oxodG and an absorbance detector for the detection of dG8-oxodG. The method is in detail described elsewhere [85].

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Lipid peroxidation

The urinary samples were analysed for free 8-iso-prostaglandin-F$_{2\alpha}$ (8-iso-PGF$_{2\alpha}$), without any extraction, by a validated radioimmunoassay (see Paper II). The urinary levels of 8-iso-PGF$_{2\alpha}$ were adjusted for creatinine concentration.

In the malondialdehyd (MDA) analyses, a thiobarbituric acid reaction was carried out by mixing phosphoric acid with water and by mixing thiobarbituric acid with plasma samples. Incubation was carried out in a boiling water bath and the mixture was cooled on ice. The malondialdehyde-thiobarbituric acid complex was extracted with methanol and quantified using a HPLC column, and analysed with a fluorescence detector.

Inflammation analyses

Interleukin-6 (IL-6) was analysed in plasma with an enzyme-linked immunosorbent assay (ELISA) kit. The colour reaction was proportional to the level of bound IL-6.

Analyses with a latex-enhanced reagent was used to perform high sensitivity CRP measurements from plasma samples.

The urinary samples were analysed for 15-keto-dihydro-PGF$_{2\alpha}$ without extraction, by a validated radioimmunoassay (see paper II). The levels were corrected for urinary creatinine.

Statistics

Statistic analyses were performed using the statistic software JMP version 3.2 (SAS Institute, Cary, N.C., USA). All correlation coefficients were calculated as Spearman´s rank correlation coefficients. Probability values of <0.01 were considered as significant for the correlation tests. This was to protect against false positive significances because of the multiple analyses in the study. In the correlation analyses of oxidative stress or inflammation, probability values of $p<0.05$ was used after correction with Bonferroni-Holm [87]. Correlations were calculated on n=54 for the whole group, n=53 for dietary data and n=51 for inflammatory data. Energy corrected dietary values (/1000 kJ) were used in all correlation analyses with dietary intake.

For many variables there was no normal distribution (Shapiro-Wilks test, W<0.95). Therefore non-parametric tests were used. Spearman rank correlation coefficients were calculated when correlations between variables were tested. Wilcoxon two-sample test was used to test differences between sexes. Probability values of <0.05 were here considered as significant. Sex differences were calculated on n=54 for the whole group, n=53 for dietary data and n=51 for inflammation data.

4. Results and discussion

4.1 Impact of a lifestyle intervention program on oxidative stress

To investigate possible changes in DNA-oxidation from a lifestyle intervention in metabolic syndrome patients, high alkaline Comet assay with FPG was used. DNA-oxidation is considered to be an important biomarker for further disease in this patient group and it has not been analysed before in such an intervention study.
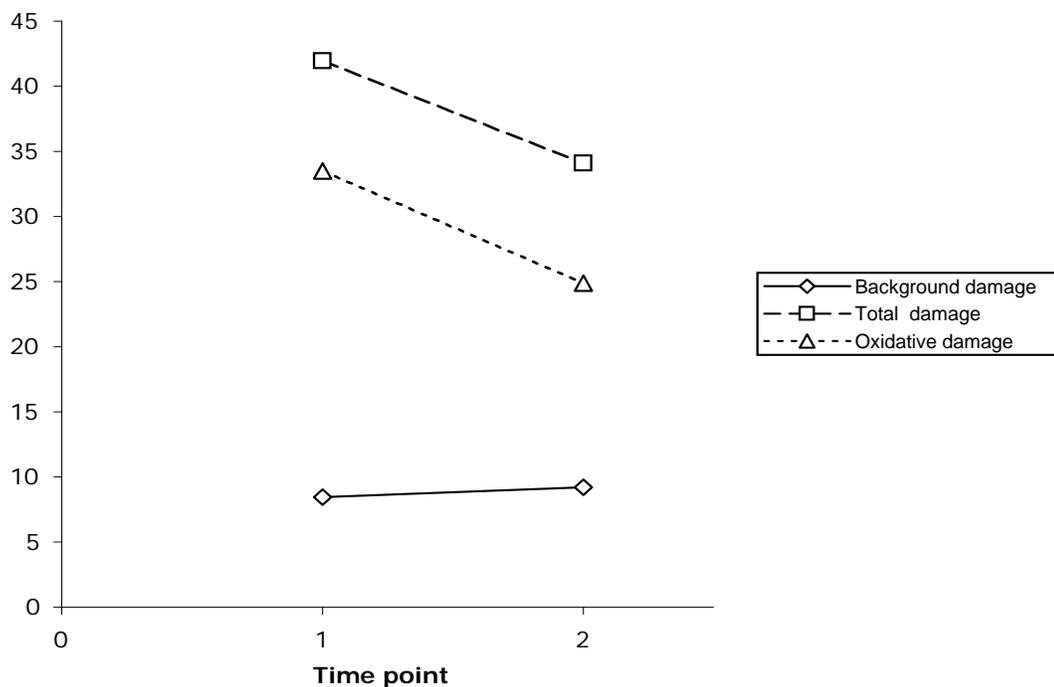
Figure 7. The background DNA damage level (single strand breaks and alkali labile sites) did not change after 6 months. The total DNA damage (background damage and oxidative damage) is statistically decreased after 6 months (P<0.001). The oxidative DNA damage level (background damage subtracted from total damage) was significantly decreased after 6 months (p<0.001). Time point 1 is at the start of the program and time point 2 is after six months in the program. The DNA damage levels were measured in % Tail.

In paper I, it was found that the DNA damage in cells from the patients after FPG-treatment (total damage) decreased significantly from 42.0 to 34.1% Tail ($p<0.001$) between the two time points, while the background DNA damage (single strand breaks and alkali labile sites) was not significantly changed (Figure 7). This was analysed with 3-way ANOVA with two levels of damage; FPG treated (background damage with oxidative damage) and not FPG treated (background damage), point of time (start and six months) and patients (n=26), the first two parameters fixed and the third random. Tukey´s test was used to make pair-wise comparisons of background damage as well as FPG treated damage over time.

The conclusion from these analyses was that the oxidative DNA damage in this group of patients decreased significantly ($p<0.001$) after the six months intervention program. After subtraction of background DNA damage from FPG-treated DNA damage (total damage), the oxidative DNA damage remains. The oxidative DNA damage decreased significantly from 33.5 to 24.9% Tail ($p<0.001$), as shown in Figure 7. Since the DNA oxidation decreased significantly in parallel with a positive change in other important risk parameters for this patient group after the lifestyle intervention program (see 4.3), the method to analyse DNA oxidation can be recommended to be included in coming similar intervention studies.

The lacto-vegetarian diet at the health resort as well as changes at home towards a healthier diet is one factor that could explain the result, since a high intake of fruit and vegetables can prevent oxidative stress, cardiovascular diseases [88](10), and diabetes type II [21]. Antioxidants in fruit and vegetables could explain these positive health effects, since several studies on humans have shown that supplementation of antioxidants can protect against oxidative stress on DNA [40, 60, 89].

The physical activities at the health resort as well as an increased physical activity at home could be another explanatory factor. Regular physical exercise and carbohydrate-rich diets also increase the resistance against oxidative stress [90], and physical activity can decrease the risk for obesity and type II diabetes in mice [49]. Psychological stress, excess alcohol intake and cigarette smoke are lifestyle factors, which increase oxidative stress [90] and are minimized during the program at the health resort. It is known that chronic alcohol exposure could damage DNA and inhibits DNA repair systems [91]. Cigarette smoking can induce ROS formation; increase oxidative- and nitric DNA lesions and chromosome damage [92, 93]. Most likely the dominating

protective factors of the intervention were a modified diet with more fruit and vegetables in combination with stress reduction that also affects the food habits.

4.2 Dietary factors in relation to oxidative stress

It has been suggested that development of diabetes type II can be reduced by increased intake of antioxidants in the diet [94]. Dietary intake of α-carotene, β-carotene and lycopene, as well as plasma β-carotene concentrations, have been shown to have beneficial associations with glucose metabolism in subjects at high risk of type II diabetes [95], and the glucose metabolism has been associated with oxidative stress [20, 76]. Correlation analyses were performed in Paper II to investigate the association between dietary antioxidants/plasma antioxidants and oxidative stress/inflammation. Inverse relations were found between fruit and vegetable intake and DNA oxidation / lipid peroxidation as shown in Figure 8.
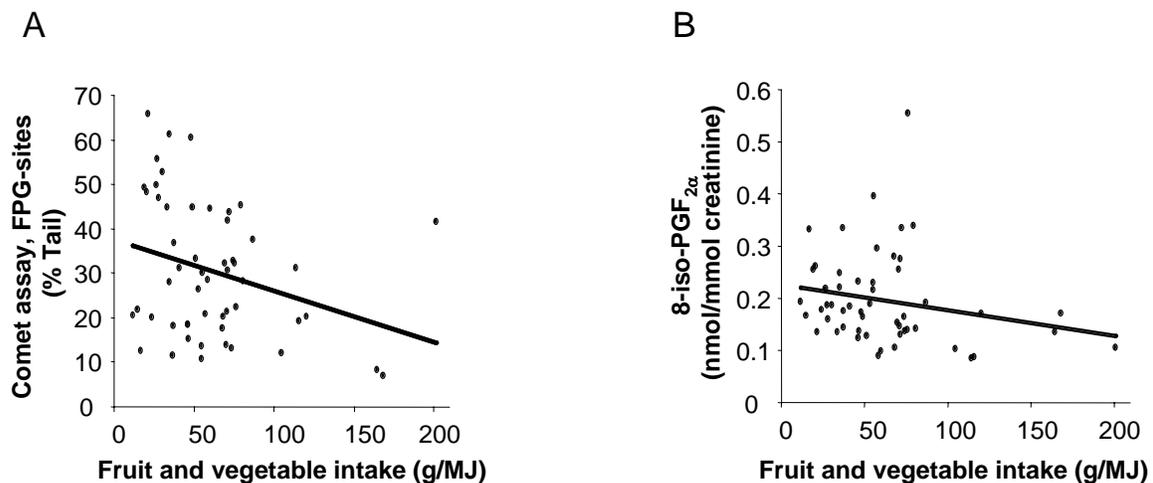

Figure 8. Fruit and vegetable intake and its relation to (A) DNA-oxidation (FPG-sites, %Tail) and to (B) lipid peroxidation (nmol 8-iso-PGF$_{2\alpha}$ /mmol creatinine). In figure A r=-0.2724 and p=0.0485 and in figure B r=-0.2774 and p=0.0443.

In this study the carotenoids were related to inflammation but not to oxidative stress. Vitamin C, on the other hand, had a negative correlation to lipid peroxidation. This might reflect that especially fruit and vegetables rich in vitamin C have the ability to protect against oxidative

stress. Furthermore, our results suggest that plasma ascorbate might protect against DNA oxidation and plasma γ-tocopherol against lipid peroxidation. We propose that dietary factors like fruit and vegetable intake and antioxidants affect the levels of oxidative stress in diabetes type II patients and this might be related to the blood glucose levels. Since this patient group have been reported to have a high DNA oxidation [17, 18] and lipid peroxidation [15, 16], and that both had inverse relationships with antioxidants in diet and plasma, it is most likely important for this group to have a diet rich in fruit and vegetables.

4.3 Anthropometry and risk factors in blood relates to oxidative stress

After the intervention in Paper I, HDL cholesterol showed significantly higher values (p<0.01) in the patients. Since HDL is the "good cholesterol" this is a positive effect of the intervention. Total cholesterol, LDL cholesterol and blood glucose were not significantly changed. Blood glucose levels in the metabolic syndrome patients in Paper I were in average between 6 and 7 mmol/L, while the type II diabetes patients in Paper II had an average of around 8 mmol/L. These are all values clinically considered in the risk zone of being too high, which is typical for these patient groups. The level of the long-term sugar biomarker HbA1c (6.1 % in average in Paper II) shows that they have well-controlled diabetes. The average insulin value of 11.5 mU/L is high, but in the normal interval for this group. It can be concluded that the blood glucose and insulin values in both Paper I and Paper II shows two groups with a rather functional metabolic control. For risk groups like these, it is considered of extra importance with a modification of lifestyle to prevent further oxidative stress, obesity, diabetes or CVD. All positive changes in relevant risk factors for metabolic syndrome patients in Paper I indicate that a lifestyle intervention program can be considered to be of importance for this patient group.

There were no significant correlations in Paper I between DNA damage values and anthropometric data or blood values on an individual level. The relatively low number of patients included in the study may explain this. However, comparing the upper and lower quartile in individual changes in oxidative stress on DNA, initial values of LDL-cholesterol and initial ratio of LDL/HDL-cholesterol were both significantly different. The most pronounced reduction in oxidative stress on DNA was in patients with a higher initial value of LDL cholesterol and a higher initial LDL/HDL ratio (Figure 9). The cholesterol type and levels might therefore be one

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factor that affects the change in oxidative stress on DNA during the intervention in the metabolic syndrome patients.
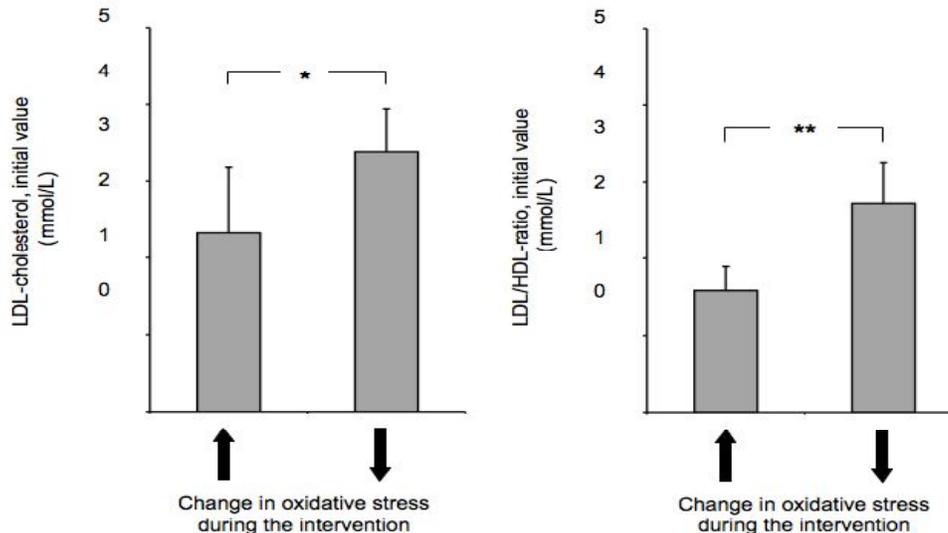

Figure 9. When comparing individual changes in oxidative stress on DNA from the intervention, the upper (↑, n=6) and lower (↓, n=6) quartile were significantly different in initial values of LDL-cholesterol (2.33 and 3.38 mmol/L, respectively, p=0.030) and initial ratio of LDL/HDL-cholesterol (1.58 and 2.72 mmol/L, respectively, p=0.0011).

Oxidative stress is a known pathogenic mechanism in diabetic complications. It has been hypothesized that insulin resistance might cause elevated concentrations of free radicals in plasma, which, in turn, might be responsible for a deterioration of insulin action, with hyperglycemia being a contributory factor [97]. The levels of blood glucose and HbA1c (long term blood sugar) was shown to be positively correlated to lipid peroxidation (Table 6) in the diabetes type II patients in Paper II [96]. High glucose levels have previously been shown to increase production of free radicals [98]. Further more, experimental and clinical data suggest an inverse association between insulin sensitivity and levels of ROS [99]. It seems that the metabolic control in these patients relates to lipid peroxidation, which could explain why patients with

metabolic syndrome and diabetes type II have higher levels of oxidative stress than healthy individuals [15,16], and that this might be mainly to hyperglycemia [20].

Table 6. Correlations between blood risk factors and anthropometry versus inflammatory biomarkers in another study on the same diabetes type II patient group as in Paper II [96].

Blood risk factors and anthropometry		Lipid peroxidation and inflammatory biomarkers	r	P
Glucose	-	8-Iso-PGF$_{2-\alpha}$	0.33	0.013
HbA1c	-	8-Iso-PGF$_{2-\alpha}$	0.29	0.030
Weight	-	CRP	0.36	0.006
BMI	-	CRP	0.32	0.016
Waist	-	CRP	0.37	0.005

P-value < 0.05 was considered as significant using Spearman rank correlation coefficient.

4.4 Fruit, vegetables and antioxidants in relation to inflammation

Specific nutrients, food and also obesity as such, have a clear impact on the development of diabetes type II and this effect may, in part, be mediated via the inflammatory status [100]. Also, a high fruit and vegetable intake has been shown to be associated with a low level of inflammation [70].

In regard to inflammation and its relation to dietary intake, a strong negative correlation between inflammation and all carotenoids measured in plasma was found in Paper II (Figure 10). This is in line with other studies showing a negative relation between carotenoids in plasma and inflammation [101] and also that plasma carotenoids have been shown to be good predictors of fruit and vegetable intake measured with food questionnaires [102]. It is therefore likely that the carotenoids in fruit and vegetables can explain the positive dietary effects on inflammation.
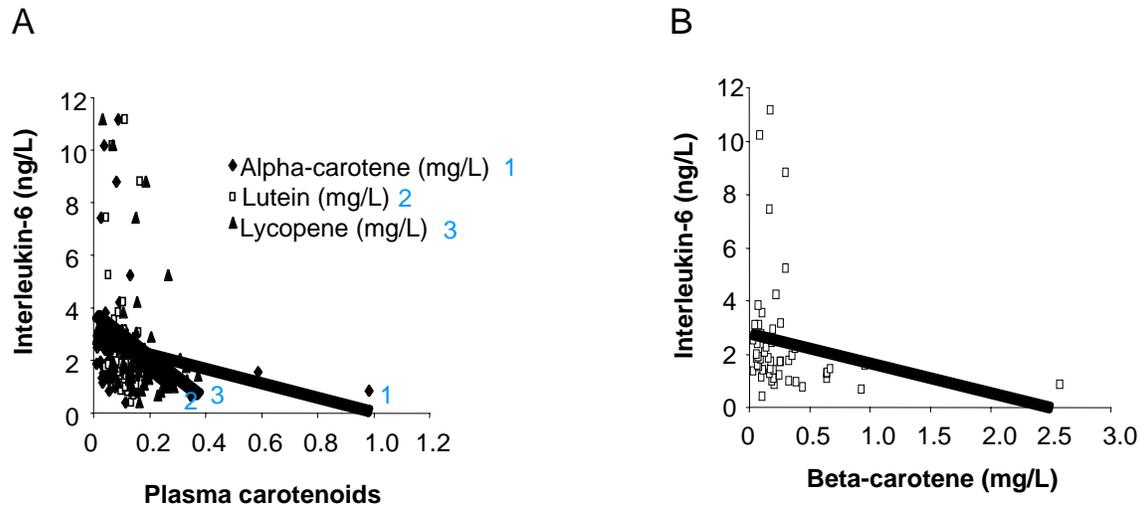
Figure 10. Correlations between plasma carotenoids (mg/L) and inflammation, IL-6 (ng/L). The carotenoids in figure A are (1) alpha-carotene (r=-0.4101, p=0.0025), (2) lutein (r=-0.2960, p=0.0331), and (3) lycopene (r=-0.2795, p=0.0448) and in figure B beta-carotene is shown (r=-0.3614, p=0.0085).

In contrast, γ-tocopherol had a positive correlation to inflammation. The patients had a high intake of α-tocopherol which can affect plasma concentrations of γ-tocopherol, due to the competition between α- and γ-tocopherol for the tocopherol binding proteins [70]. Therefore no clear conclusions can be drawn from the γ-tocopherol correlations.

4.5 Blood risk factors and anthropometry relates to inflammation

Abdominal obesity has been shown to be associated with inflammation (CRP) in diabetes type II patients [96]. Metabolic syndrome patients have higher acute-phase inflammation (CRP, IL-6), and this seems to follow the grade of metabolic syndrome [103]. Pro-inflammatory cytokines such as IL-6 and TNF-α are also known to cause insulin resistance and impair insulin secretion, resulting in the physiologically appropriate hyperglycemia of the stress response, and in the long term to a chronic inflammatory response and further diseases like metabolic syndrome or diabetes. Furthermore, a wide range of risk factors for diabetes type II are also known to be associated with an augmented acute-phase inflammatory response, among them obesity and

physical inactivity [103]. This supports data from diabetes type II patients (Table 6) that weight, BMI and waist circumference correlates positively with inflammation [96]. Besides this, it is known that obesity increases the risk of diabetes and CVD [12] and especially waist circumference has the same impact as BMI to identify risk for decreased insulin sensitivity and CVD [13].

4.6 Levels of oxidative stress and inflammation in diabetes type II patients

Comparing the values of oxidative stress on DNA in Paper II (Table 7) with a healthy population [7, 104], 8-oxodG levels were similar, but values from the Comet assay were much higher in the patient group with diabetes type II. Since the Comet assay is a more sensitive measurement of oxidative DNA-damage and also gives a broader picture of the DNA-oxidation, it seems that this group of patients generally had a high DNA-oxidation. The lack of difference between patients and healthy subjects in 8-oxodG level, could also be due to the high adventitious oxidation that is known to occur during sample preparation when analysing 8-oxodG with HPLC.

The lipid peroxidation in Paper II (8-Iso- $PGF_{2-\alpha}$) was slightly higher than in healthy subjects [105] and diabetes type II patients have previously been shown to have an increased lipid peroxidation [99, 106]. The females in Paper II had higher levels of (8-Iso- $PGF_{2-\alpha}$) than the men, $p<0.05$ (Table 7), which might mean that the females were less healthy than the men.

Table 7. Levels of biomarkers for oxidative stress and inflammation in Paper II.

Oxidative stress and inflammation biomarkers	Mean $\pm$ SD	Median	Range
8-hydroxy-deoxyguanosine (8-oxodG/10^6 dG)	0.97 ± 0.45	0.88	0.20-2.97
Comet assay (% tail Fpg-sites)	30.2 ± 15.1	28.37	6.85-65.6
Malondialdehyd (mmol/L)	0.68 ± 0.08	0.66	0.52-0.85
8-Iso- $PGF_{2-\alpha}$ (nmol/mmol creatinine) ‡	0.19 ± 0.09	0.17	0.06-0.56
CRP (mg/L), only patients with CRP<10	2.50 ± 2.46	1.8	0.16-9.53
CRP (mg/L), all patients	3.15 ± 4.30	1.8	0.16-27.6
Interleukin-6 (ng/L) ‡ §	2.50 ± 2.20	1.8	0.40-11.20
15-keto-dihydro-$PGF_{2-\alpha}$ (nmol/mmol creatinine)	0.24 ± 0.10	0.24	0.08-0.68

‡ Significant differences between gender using unpaired t-test or Wilcoxon 2-sample test, $p<0.05$.
§ Significant differences between non smokers and smokers using unpaired t-test or Wilcoxon 2-sample test, $p<0.05$.

Markers for inflammation were measured in the patients in Paper II, and the levels are presented in Table 7. The serum levels of CRP were in a wide range in the patient group. Two patients had very high CRP-values i.e. had a high acute inflammation. A group excluding patients with CRP levels above 10 mg/L was investigated to be able to see correlations of inflammation with diet and plasma antioxidants (see below), without interaction of these high acute inflammation values. This new group had a more normal mean value of 2.50 mg/L.

Furthermore, the oxidation product of prostaglandin 15-keto-dihydro-PGF$_{2-\alpha}$ (mean 0.24 nmol/mmol creatinine) was in an expected normal value range [105]. For IL-6, the levels were in the higher range (mean 2.5 ng/L), and significantly higher in the men, $p<0.05$ (Table 7). Earlier reported levels in elderly diabetic men between 2.47-3.57 ng/L [107], can be compared to the men in the present study who had a mean value of 3.22 ng/L. Plasma IL-6, BMI and adiposity are closely associated [108], which indicates a link between inflammation (IL-6) and metabolic syndrome in diabetes type II patients. It can also be reported that the smokers had significantly higher values of IL-6 than the non-smokers (Table 7), and that smoking is a risk factor connected to inflammation, oxidative stress and obesity.

4.7 Dietary factors in diabetes type II patients.

In Paper II, fruit and vegetable intake was analysed for a group of diabetes type II patients. Comparisons with the dietary recommendations for the Northern countries [35] and also to a Swedish national dietary survey [34] were done. The energy intake seen in Paper II (8.6 MJ/d) followed the recommendations in the Nordic nutrition recommendations (NNR) of >8.0 MJ/d [35], and therefore the intake of nutrients in the diabetes type II patient group are comparable to the recommendations. Usually a time period of 4 days is considered necessary to definitely judge the level of energy intake [109], but the 2 weekdays and 1 weekend day model used in Paper II is considered enough to roughly estimate the dietary intake on a group level. The days of the week were also randomly represented among the participants. The energy intake of fats (31.2 E%) and proteins (17.4 E%) were quite similar to the 34 E% fats and 16 E% proteins in the Swedish population [34] and were comparable to the recommended 30 E% and 15 E% respectively [35]. These levels and comparisons is presented in Table 8.

The micronutrients in the food were also at recommended levels, except for vitamin E, folate and selenium for men, which should be higher according to the recommendations (Table 8). The recommended intake of fruit and vegetables in Sweden is 500g/woman/day and 700g/man/day, and the diabetes type II group in Paper II had close to recommended levels of fruit and vegetable intake for women (490 g/day) and for men (516 g/day), which is far above the national level for Swedish men (Table 8). No gender differences could be seen in the dietary intake, when it had been corrected for energy intake, except for B12, which was higher for women (Table 8). Dietary intake of the antioxidants β-carotene, vitamin E, α-tocopherol, vitamin C and folate were positively related to the intake of fruit and vegetables ($p<0.01$, not shown), which was as expected since we get all these antioxidants, except for α-tocopherol, mainly from fruit and vegetables. Furthermore, plasma levels of alpha-carotene and Beta-carotene had strong positive relations to fruit and vegetable intake (see Paper II), which is supported in a large European study (102). Alpha- and Beta-carotene in plasma can therefore be considered good biomarkers for fruit and vegetable intake.

Table 8. Comparison of the dietary intake in the diabetes type II patients in Paper II (28 females and 25 males) with a Swedish national survey "Riksmaten" (626 females and 589 males) and the Nordic nutrition recommendations NNR.

Dietary intake	Paper II Females	Paper II Males	Riksmaten Females	Riksmaten Males	NNR Females	NNR Males
Energy	8.2 MJ	8.7 MJ	7.8 MJ	9.9 MJ	> 8.0 MJ	> 8.0 MJ
Proteins	17.7 E%	17.1 E%	16 E%	16 E%	15 E%	15 E%
Fats E%	32.0 E%	30.3 E%	34 E%	34 E%	30 E%	30 E%
Carbohydrates	47.9 E%	48.4 E%	47 E%	46 E%	55 E%	55 E%
Betacarotene	4103 RE	3320 RE	1874 μg	1708 μg	700 RE	900 RE
Vitamin E	10.3 a-TE	9.00 a-TE	-	-	8.0 a-TE	10.0 a-TE
α-tocopherol	9.79 mg	8.49 mg	6.8 mg	7.9 mg	-	-
Vitamin C	137.4 mg	143.6 mg	93 mg	180 mg	75 mg	75 mg
Vitamin B12	7.78 μg*	6.26 μg	6.0 μg	6.9 μg	2.0 μg	2.0 μg
Zinc	11.9 mg	12.6 mg	9.9 mg	12.6 mg	7.0 g	9.0 g
Folate	306.3 μg	284.4 μg	217 μg	232 μg	300 μg	300 μg
Selen	42.1 μg	46.6 μg	32 μg	36 μg	40.0 μg	50.0 μg
Fruit and vegetable intake	490.5 g	515.9 g	261 g	188 g	500 g	700 g

* Significally higher for females, $p<0.05$.

5. Conclusions

A 17-day long lifestyle intervention program with a 5-day follow up after three months had the impact to lower the oxidative stress on DNA, in a group of metabolic syndrome patients, six months after start. The most pronounced reduction in oxidative stress on DNA after the lifestyle intervention was in patients with a higher initial value of LDL cholesterol and a higher initial LDL/HDL ratio. Also other relevant risk factors for metabolic syndrome patients i.e. BMI, waist circumference, body fat and HDL cholesterol were positively affected by the lifestyle intervention program. We therefore conclude that lifestyle interventions might be important for metabolic syndrome patients. We also suggest oxidative stress on DNA to be an important biomarker to evaluate such lifestyle interventions.

Furthermore, investigations on how dietary factors and also blood risk factors and anthropometry relate to oxidative stress and inflammation in a group of diabetes type II patients gives us the following conclusions:

A high intake of fruit and vegetables as well as vitamin C, and also high levels of plasma ascorbate, might decrease oxidative stress in diabetes type II patients. A decreased oxidative stress in this group prevents further progress of the disease and secondary complications. Intake of fruit and vegetables, especially those rich in vitamin C and perhaps also dietary supplementation with vitamin C, will possibly have health advantages for the patients by lowering their oxidative pressure on DNA and lipids.

High levels of plasma carotenoids, which have shown to be good markers for fruit and vegetable intake, might decrease inflammation. It is suggested that these factors can be used as a complementary treatment to decrease the higher inflammation levels seen in diabetes type II patients.

Plasma values of α-carotene and β-carotene can be considered as good biomarkers for fruit and vegetable intake. This can be of use in combination with food diaries or other methods in studies analysing intake of fruits and vegetables and antioxidants.

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