UNIVERSITY OF COPENHAGEN

Glycaemic status in relation to oxidative stress and inflammation in well-controlled type 2 diabetes subjects

Rytter, Elisabet; Vesseby, Bengt; Åsgård, Rikard; Johansson, Clara; Sjödin, Anders Mikael; Abramsson-Zetterberg, Lilianne; Möller, Lennart; Basu, Samar

Citation for published version (APA):
Rytter, E., Vesseby, B., Åsgård, R., Johansson, C., Sjödin, A. M., Abramsson-Zetterberg, L., ... Basu, S. (2009). Glycaemic status in relation to oxidative stress and inflammation in well-controlled type 2 diabetes subjects. *British Journal of Nutrition*, *101*, 1423-1426. https://doi.org/10.1017/S0007114508076204

Short Communication

Glycaemic status in relation to oxidative stress and inflammation in well-controlled type 2 diabetes subjects

Elisabet Rytter[1], Bengt Vessby[1], Rikard Åsgård[2], Clara Johansson[2], Anders Sjödin[3], Lilianne Abramsson-Zetterberg[4], Lennart Möller[2] and Samar Basu[1]*

[1]*Clinical Nutrition and Metabolism, Department of Public Health and Caring Science, Uppsala University, Uppsala, Sweden*
[2]*Department of Biosciences and Nutrition, Karolinska Institute, Huddinge, Sweden*
[3]*Department of Human Nutrition, Copenhagen University, Copenhagen, Denmark*
[4]*National Food Administration, Uppsala, Sweden*

The aim of the present observational study was to investigate the relationships between glycaemic status and levels of oxidative stress and inflammation in well-controlled type 2 diabetes subjects. Metabolic variables (weight, BMI, waist circumference (waist), blood glucose, glycated Hb (HbA$_{1c}$), insulin, blood lipids), biomarkers of oxidative stress (8-iso-PGF$_{2\alpha}$, malondialdehyde, 8-oxo-7,8-dihydro-2′-deoxyguanosine, formamido pyrimidine glycosylase-sites, frequency of micronucleated erythrocytes, nitrotyrosine) and inflammatory markers (high sensitivity C-reactive protein (hsCRP), IL-6, cyclo-oxygenase-catalyzed PGF$_{2\alpha}$-metabolite) were measured. Fifty-six patients (thirty women and twenty-six men, age 62·3 (SD 7·0) years, HbA$_{1c}$ 6·1 (SD 0·9) %, BMI 28·3 (SD 3·8) kg/m^2, waist 99·6 (SD 11·1) cm) were included in the study. HbA$_{1c}$ (r 0·29, $P=0·03$) and blood glucose (r 0·33, $P=0·01$) correlated positively with 8-iso-PGF$_{2\alpha}$. Positive correlations were also observed between HbA$_{1c}$ and nitrotyrosine (r 0·42, $P=0·01$), waist and hsCRP (r 0·37, $P=0·005$), hsCRP and IL-6 (r 0·61, $P<0·0001$) and between PGF$_{2\alpha}$-metabolite and 8-iso-PGF$_{2\alpha}$ (r 0·27, $P=0·048$). The present study indicates that glycaemic status is associated with oxidative stress even in subjects with well-controlled type 2 diabetes. Furthermore, inflammation was more related to abdominal obesity than to glycaemic control. A large number of biomarkers of oxidative stress and inflammation were investigated, but only a few associations were found between the markers. This could be due to the fact that none of these biomarkers biosynthesises via similar pathways or simultaneously owing to their diverse nature and origin.

Glycaemic control: Oxidative stress: Inflammation: Diabetes mellitus type 2

Diabetes is a disorder associated with an increased risk of developing vascular and other health complications. Oxidative stress and inflammation are the major pathogenetic mechanisms considered to be implicated in these complications[1,2]. Subjects with type 2 diabetes have been shown to have increased levels of lipid peroxidation, oxidative damage to DNA and protein oxidation[1], presumably caused by an overproduction of free radicals and a decreased antioxidative defence. Enhanced production of free radicals is related to hyperglycaemia, insulin resistance and hyperinsulinaemia[3]. High levels of glucose lead to an increased production of free radicals via different mechanisms such as glucose autooxidation and formation of advanced glycation end products[4].

Besides oxidative stress, inflammation is also implicated in the development of complications in type 2 diabetes[2]. Cyclo-oxygenase catalyzed PG formation, and subsequently low-grade inflammation is suggested to be an early event in the development of type 2 diabetes that is further linked to oxidative stress[5]. Elevated levels of high sensitivity C-reactive protein (hsCRP) and IL-6 are seen in subjects with type 2 diabetes[6], and are also associated with an increased risk for developing the disease in future[7]. However, whether inflammation and oxidative stress are related to glycaemic control is still not fully clarified.

The aim of the present observational study was to investigate the relationships between glycaemic control and levels of oxidative stress and inflammation in subjects with well-controlled type 2 diabetes.

Experimental methods

Subjects and study design

Participants were recruited to take part in an intervention study with antioxidant supplementation. Baseline results are described in the present article and results from the intervention study are

presented elsewhere. Inclusion criteria were age 40–75 years, type 2 diabetes treated with either diet or diet and oral hypoglycaemic medication, glycated Hb (HbA_{1c}) < 10 % and BMI < 35 kg/m^2. Exclusion criteria were insulin-dependent diabetes, known CVD, acute inflammatory, liver, kidney or thyroid diseases as well as medication or supplementation that could affect oxidative or inflammatory status. Subjects gave their written consent to participate in the study. The study was approved by the Ethical Committee of the Medical Faculty at Uppsala University, Sweden.

Blood and urine sample were drawn in the morning after an overnight fast. Body height, weight, waist circumference (waist) and blood pressure were recorded at the same time.

Laboratory analysis

Blood glucose concentration was analyzed by an enzymatic technique (HemoCue). HbA_{1c} was analyzed with high performance liquid chromatography. Plasma insulin was assayed with an enzymatic immunological assay (Mercodia, Uppsala, Sweden) in a Coda Automated EIA Analyzer (Bio-Rad Laboratories, Hercules, CA, USA). Serum cholesterol, HDL- cholesterol and TAG concentrations were analysed by enzymatic colorimetric methods (Thermo Electron Corporation, Vantaa, Finland) in a Konelab 20 Clinical Chemistry Analyzer (Thermo Electron Corporation). LDL- cholesterol was calculated according to Friedewald[8].

Biomarkers of oxidative stress

Comet assay and 8-oxo-7,8-dihydro-2′-deoxyguanosine. A high-alkaline formamido pyrimidine glycosylase (FPG) version of the comet assay[9] was used with some modifications. For the 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) analyses, DNA was isolated using a cold work-up procedure[10] with small modifications, followed by enzymatic hydrolysis[11]. The amount of 8-oxodG per undamaged 2′-deoxyguanosine was analysed using on-line electrochemical and uv detection after separation of the nucleosides with HPLC.

Frequency of micronucleated erythrocytes

A flow cytometry-based micronucleus assay in very young erythrocytes from humans, transferrin-positive reticulocytes[12], was used. In this micronucleus assay, measuring the frequency of micronucleus-transferrin-positive reticulocytes (fMN-Trf-Ret), the very young erythrocytes were separated from the mature before analysis. The detection limit was approximately a difference of 0·2 of the background fMN-Trf-Ret. The background fMN-Trf-Ret was approximately 1‰.

Malondialdehyde

Plasma malondialdehyde (MDA) concentration was measured by HPCL and fluorescence detection as earlier described[13].

F_2-isoprostanes

Free 8-iso-PGF$_{2\alpha}$, a major F_2-isoprostane in urine, was analysed by a validated RIA developed by Basu[14]. Levels were adjusted for urinary creatinine concentration.

Nitrotyrosine

Nitrotyrosine was assayed in plasma using a commercially available enzymatic immunological assay (Bioxytech, Oxis-Research, Portland, OR, USA).

Biomarkers of inflammation

High-sensitivity C-reactive protein. High-sensitivity C-reactive protein measurement was performed in plasma by a latex-enhanced reagent (Dade Behring, Deerfield, IL, USA) with the use of a Behring BN ProSpec analyzer (Dade Behring).

Interleukin-6

IL-6 was analysed in plasma by a high-sensitivity ELISA kit (IL-6 HS, R&D Systems, Minneapolis, MN, USA). Samples and standards were pipetted in a microtitre plate coated with monoclonal antibody against IL-6. After incubation and washing enzyme substrate solution was pipetted and followed by anti-IL-6 antibody. The colour reaction was proportional to the bound IL-6.

Prostaglandin $F_{2\alpha}$-metabolite

Urinary 15-keto-dihydro-PGF$_{2\alpha}$, a major metabolite of primary PGF$_{2\alpha}$, was analysed, by a validated RIA developed by Basu[15]. Levels were corrected for urinary creatinine concentration.

Statistical analysis

The statistical software JMP version 3.2 (SAS Institute, Cary, NC, USA) was used. Probability values < 0·05 were regarded as statistically significant. The unpaired t test or the Wilcoxon two-sample test was used to analyse sex differences. The correlation coefficients (Pearson's or Spearman's coefficients) were calculated when analysing correlations.

Results

Clinical characteristic

Fifty-six participants (thirty women and twenty-six men, forty-eight non-smokers and eight smokers) were included in the study. Significant differences between sexes were found for weight (men > women), cholesterol, HDL- and LDL-cholesterol (women > men), 8-iso-PGF$_{2\alpha}$ and nitrotyrosine (women > men) and IL-6 (men > women). Smokers had a higher IL-6 level compared with non-smokers and almost every biomarker for oxidative stress and inflammation tended to be higher in smokers. Twenty-one persons were treated with diet only, thirty-five with the addition of anti-diabetic medication (sulfonylureas or other insulin-stimulating compounds or/and biguanides). Baseline characteristics are presented as means and standard deviations: age 62·3 (SD 7·0)

years; BMI 28·3 (SD 3·8) kg/m^2; weight 81·9 (SD 13·8) kg; waist 99·6 (SD 11·1) cm; systolic blood pressure 143 (SD 14) mmHg; diastolic blood pressure 78 (SD 9) mmHg; HbA$_{1c}$ 6·1 (SD 0·9)%; fasting blood glucose 7·8 (SD 2·3) mmol/l; insulin 69·3 (SD 40·2) pmol/l; TAG 1·7 (SD 0·9) mmol/l; LDL-cholesterol 2·9 (SD 0·9) mmol/l; HDL-cholesterol 1·1 (SD 0·2) mmol/l; cholesterol 4·7 (SD 1·0) mmol/l; 8-oxodG/10^6 dG 0·96 (SD 0·45); FPG-sites 30·2 (SD 15·1) % tail; fMN-Trf-Ret 0·90 (SD 0·50) ‰; MDA 0·68 (SD 0·08) μmol/l; 8-iso-PGF$_{2\alpha}$ 0·19 (SD 0·09) nmol/mmol creatinine; nitrotyrosine 245 (SD 401) nmol/l; hsCRP 3·1(SD 4·2) mg/l; IL-6 2·5 (SD 2·2) ng/l; 15-keto-dihydro-PGF$_{2\alpha}$ 0·24 (SD 0·10) nmol/mmol creatinine.

Relationships between glycaemic control and indicators of oxidative stress and inflammation

Urinary 8-iso-PGF$_{2\alpha}$ was positively correlated to fasting blood glucose (r 0·33, $P=0.01$) as well as HbA$_{1c}$ (r 0·29, $P=0.03$). In addition, there was a positive correlation between HbA$_{1c}$ and nitrotyrosine (r 0·43, $P=0.01$). A negative correlation was seen between HbA$_{1c}$ and MDA (r −0·32, $P=0.017$). Waist (r 0·37, $P=0.005$), BMI (r 0·32, $P=0.016$) and weight (r 0·36, $P=0.006$) correlated positively with hsCRP. Correlations were found between 8-iso-PGF$_{2\alpha}$ and MDA (r −0·33, $P=0.012$), hsCRP and IL-6 (r 0·61, $P<0.0001$) and 8-iso-PGF$_{2\alpha}$ and 15-keto-dihydro-PGF$_{2\alpha}$ (r 0·27, $P=0.048$).

Excluding smokers (n 8) from the correlation analyses decreased power of the study but did not change main results. No change in main results of correlation analyses was found when studying the subjects treated with diet only or treated with diet plus diabetic medication. An exception was a positive association between FPG-sites and blood glucose (r 0·7, $P=0.0004$) in subjects treated only with diet.

Discussion

The study showed positive associations between glycaemic control (blood glucose and HbA$_{1c}$) and urinary 8-iso-PGF$_{2\alpha}$ and HbA$_{1c}$ and nitrotyrosine, demonstrating a significant biological relationship between glycaemic control and oxidative stress. It was also found that abdominal obesity and low-grade inflammation (hsCRP) were closely related to each other. Despite the inclusion of a considerable number of biomarkers of oxidative stress and inflammation, only a few associations were found among these markers.

To the best of our knowledge, no other study investigating subjects with type 2 diabetes has reported so many biomarkers of oxidative stress and inflammation simultaneously. Totally six different biomarkers for oxidative stress and three for inflammation were measured in this patient group in order to clarify the relationships between glycaemic control, oxidative stress and inflammation since the latter are two vital pathologies that are considered to be the integrated parts of the metabolic syndrome[6,16].

Glycaemic control related to oxidative stress

The direct relationship between glycaemic control and oxidative stress found in the present study has also been shown elsewhere. One report described a highly significant correlation between blood glucose and urinary 8-iso-PGF$_{2\alpha}$ and a reduction of 8-iso-PGF$_{2\alpha}$ associated with improved glycaemic control[17]. However, there are also investigations not showing such association[5]. These differences could be explained by various levels of glycaemic control in the patient groups. The negative correlation between HbA$_{1c}$ and MDA found in the present study was opposite to findings by Altomare et al., who observed a positive correlation in patients with type 2 diabetes[17]. MDA is generally considered as a less specific marker of oxidative stress than the most reliable indicator of oxidative stress, 8-iso-PGF$_{2\alpha}$[18].

As far as we know, the correlation shown between HbA$_{1c}$ and nitrotyrosine has not been shown elsewhere. However, the lack of such correlation has been reported previously by Ceriello et al. [19], who at the same time observed a direct correlation between plasma glucose and nitrotyrosine.

In the present study, we found a lack of associations between glycaemic control and oxidative stress as measured by 8-oxodG and FPG-sites. Hinokio et al. [20] reported a positive correlation between glycaemic control and 8-oxodG (in urine and blood mononuclear cells) in diabetic subjects, maybe due to the higher level of HbA$_{1c}$ compared with the present study. The positive relationship between glucose and FPG-sites found in the present study in subjects treated with diet only has also been seen by other investigators[21]. To our knowledge, the present study is the first one investigating the fMN-Trf-Ret in subjects with type 2 diabetes. Healthy subjects have earlier been examined[22] with the same method and showed a similar level of frequency as in the present study.

Glycaemic control and inflammation

No correlations were found between markers of glycaemic control and inflammation in the present study as supported by Pickup et al. [23] but contradictory to observations by Ford[24]. The association found between diabetes and CRP disappeared when adjusted for BMI and waist, indicating that inflammation is more related to obesity than features for diabetes[5].

The well-known relationship between obesity and low-grade inflammation was also observed in the present study. A highly significant correlation between hsCRP and obesity was found, which also has been shown by others in subjects with type 2 diabetes[24]. A strong association between IL-6 and CRP was also observed in the present study. The positive correlation between the cyclo-oxygenase-mediated inflammatory marker PGF$_{2\alpha}$-metabolite and the oxidative stress biomarker 8-iso-PGF$_{2\alpha}$ found in the present study has also been observed by others[5], showing a link between free radical generation and inflammatory response.

Limitations of study

A limitation of the present study was the absence of a healthy reference population. Comparisons regarding oxidative stress and inflammation therefore had to be made with other investigations but with care since study conditions and methodology could differ in many aspects, especially grade of

glycaemic control and obesity. Furthermore, the present study did not measure the glucose tolerance or record the diabetes duration, parameters that could have impact on the investigated relationships between metabolic disorders, oxidative stress and inflammation in subject with diabetes.

In conclusion, the present study indicated that glycaemic status was associated with oxidative stress even in well-controlled diabetes subjects. Furthermore, inflammation was more related to abdominal obesity than to glycaemic control. The relatively small study group with a well-controlled type 2 diabetes and moderate obesity, giving a narrow range of HbA_{1c}, blood glucose, waist and BMI, increased the probability that existing relationships may not have been detected. In spite of these limitations, interesting relationships were found.

A large number of biomarkers of oxidative stress and inflammation were investigated, but only a few associations were found between the markers. This could be due to the fact that none of these biomarkers biosynthesizes via similar pathways or simultaneously owing to their diverse nature and origin.

Acknowledgements

References

1. Jialal I, Devaraj S & Venugopal SK (2002) Oxidative stress, inflammation, and diabetic vasculopathies: the role of alpha tocopherol therapy. *Free Radic Res* **36**, 1331–1336.

2. Pickup JC (2004) Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. *Diabetes Care* **27**, 813–823.

3. Ceriello A (2000) Oxidative stress and glycaemic regulation. *Metabolism* **49**, 27–29.

4. Bonnefont-Rousselot D (2002) Glucose and reactive oxygen species. *Curr Opin Clin Nutr Metab Care* **5**, 561–568.

5. Helmersson J, Vessby B, Larsson A, *et al.* (2004) Association of type 2 diabetes with cyclooxygenase-mediated inflammation and oxidative stress in an elderly population. *Circulation* **109**, 1729–1734.

6. Pickup JC, Mattock MB, Chusney GD, *et al.* (1997) NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. *Diabetologia* **40**, 1286–1292.

7. Duncan BB, Schmidt MI, Pankow JS, *et al.* (2003) Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. *Diabetes* **52**, 1799–1805.

8. Friedewald WT, Levy RI & Fredrickson DS (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* **18**, 499–502.

9. Gedik CM & Collins A (2005) Establishing the background level of base oxidation in human lymphocyte DNA: results of an interlaboratory validation study. *FASEB J* **19**, 82–84.

10. Hofer T & Moller L (2002) Optimization of the workup procedure for the analysis of 8-oxo-7,8-dihydro-2′-deoxyguanosine with electrochemical detection. *Chem Res Toxicol* **15**, 426–432.

11. Nagy E, Johansson C, Zeisig M, *et al.* (2005) Oxidative stress and DNA damage caused by the urban air pollutant 3-NBA and its isomer 2-NBA in human lung cells analyzed with three independent methods. *J Chromatogr B Anal Technol Biomed Life Sci* **827**, 94–103.

12. Abramsson-Zetterberg L, Durling LJ, Yang-Wallentin F, *et al.* (2006) The impact of folate status and folic acid supplementation on the micronucleus frequency in human erythrocytes. *Mutat Res* **603**, 33–40.

13. Ohrvall M, Tengblad S, Ekstrand B, *et al.* (1994) Malondialdehyde concentration in plasma is inversely correlated to the proportion of linoleic acid in serum lipoprotein lipids. *Atherosclerosis* **108**, 103–110.

14. Basu S (1998) Radioimmunoassay of 8-iso-prostaglandin F2alpha: an index for oxidative injury via free radical catalysed lipid peroxidation. *Prostaglandins Leukot Essent Fatty Acids* **58**, 319–325.

15. Basu S (1998) Radioimmunoassay of 15-keto-13,14-dihydro-prostaglandin F2alpha: an index for inflammation via cyclooxygenase catalysed lipid peroxidation. *Prostaglandins Leukot Essent Fatty Acids* **58**, 347–352.

16. Esposito K, Ciotola M, Schisano B, *et al.* (2006) Oxidative stress in the metabolic syndrome. *J Endocrinol Invest* **29**, 791–795.

17. Altomare E, Vendemiale G, Chicco D, *et al.* (1992) Increased lipid peroxidation in type 2 poorly controlled diabetic patients. *Diabete Metab* **18**, 264–271.

18. Basu S (2004) Isoprostanes: novel bioactive products of lipid peroxidation. *Free Radic Res* **38**, 105–122.

19. Ceriello A, Mercuri F, Quagliaro L, *et al.* (2001) Detection of nitrotyrosine in the diabetic plasma: evidence of oxidative stress. *Diabetologia* **44**, 834–838.

20. Hinokio Y, Suzuki S, Hirai M, *et al.* (1999) Oxidative DNA damage in diabetes mellitus: its association with diabetic complications. *Diabetologia* **42**, 995–998.

21. Dincer Y, Akcay T, Alademir Z, *et al.* (2002) Assessment of DNA base oxidation and glutathione level in patients with type 2 diabetes. *Mutat Res* **505**, 75–81.

22. Abramsson-Zetterberg L, Zetterberg G, *et al.* (2000) Human cytogenetic biomonitoring using flow-cytometric analysis of micronuclei in transferrin-positive immature peripheral blood reticulocytes. *Environ Mol Mutagen* **36**, 22–31.

23. Pickup JC, Chusney GD, Thomas SM, *et al.* (2000) Plasma interleukin-6, tumour necrosis factor alpha and blood cytokine production in type 2 diabetes. *Life Sci* **67**, 291–300.

24. Ford ES (1999) Body mass index, diabetes, and C-reactive protein among U.S. adults. *Diabetes Care* **22**, 1971–1977.