Inflammation, coagulation, endothelial dysfunction and oxidative stress in prediabetes — Biomarkers as a possible tool for early disease detection for rural screening

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A R T I C L E   I N F O

Article history:
Received 20 November 2014
Received in revised form 24 February 2015
Accepted 26 February 2015
Available online 6 March 2015

Keywords:
Prediabetes
Oxidative stress
Inflammation
Coagulation
Endothelial dysfunction

A B S T R A C T

Objectives: This study aims to increase understanding of the connection between oxidative stress and inflammation in diabetes disease progression to provide a basis for investigating improved diagnostic possibilities, treatment and prevention of prediabetes.

Design and methods: Differences in the level of biochemical markers of oxidative stress (erythrocyte GSH/GSSG and urinary 8-isoprostane), inflammation (CRP, IL-6), endothelial dysfunction (plasma homocysteine, urinary 8-hydroxy-2-deoxy-guanosine) and coagulation/fibrinolysis (C5a, D-Dimer) were determined in prediabetes and control subjects.

Results: While no difference was found in the 8-isoprostane levels between the two groups, the erythrocyte GSH/GSSG ratio was significantly reduced in the prediabetes group compared to control, indicating increased oxidative stress in the prediabetic state. Both urinary 8-OHdG and surprisingly also plasma homocysteine were significantly elevated in the prediabetes group, indicating endothelial dysfunction. The inflammation markers were slightly elevated in the prediabetic subjects and the same trend was found for the coagulation/fibrinolysis markers C5a and D-Dimer. These results were however not significant.

Conclusions: The small elevation of blood glucose levels in the prediabetic state may have a detectable influence on endothelial function as indicated by changes to 8-OHdG, indicating an increased DNA-damage and homocysteine release from endothelial cells. Increased oxidative stress as indicated by the reduced GSH/GSSG ratio is likely to be the link between the moderate hyperglycaemia in prediabetes and pathological changes in endothelial function, which in the long-term may promote atherogenesis and result in the development of cardiovascular disease. Early detection of prediabetes is essential to avoid diabetes development and the associated complications like cardiovascular disease. The GSH/GSSG ratio and biomarkers like urinary 8-OHdG and plasma homocysteine offer a possible tool for the assessment of prediabetes in prevention screenings.

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Introduction

Diabetes mellitus is a global health problem affecting more than 6% of the world population and its prevalence is estimated to increase to about 552 million in 2030 [1]. These numbers do not include the amount of people with prediabetes, of which 90% is unaware of their situation [2]. The resulting socio-economic burden calls for additional diagnostic and therapeutic strategies to prevent the onset of diabetes efficiently.

Diabetes mellitus, which is defined as a fasting blood glucose level of greater than 6.9 mmol/L [3] is associated with extensive organ dysfunction including diabetic retinopathy, kidney disease and cardiovascular disease (CVD), gastrointestinal disturbance, sexual dysfunction and diabetic neuropathy [4,5]. The fatal macrovascular complications account for the majority of deaths among patients with diabetes mellitus [6].

A fact to take into consideration when it comes to primary prevention of CVD is that cardiovascular risk is already increased in people with impaired glucose tolerance [7], i.e. in the prediabetic state, which is defined by an impaired fasting blood glucose level of 5.6–6.9 mmol/L [3]. Studies show that impaired fasting glucose is not just a precursor of diabetes, but an individual risk factor for mortality [8]. The prevalence of prediabetes is
increasing worldwide to probably more than 400 million cases in 2030 and, if untreated, will progress to diabetes and the associated complications [7]. The key to prevention of diabetes development and its vascular complications are therefore early detection and treatment of prediabetes.

A major part of understanding disease progression is to unravel pathological processes associated with asymptomatic or preclinical stages of disease. For screening of preclinical presentations of disease a number of criteria as recommended by the World Health Organization (WHO) have to be met. These include: (1) The condition being screened for should be an important health problem, (2) There should be a detectable early stage, (3) Treatment at an early stage should be more benefit than at a later stage, and (4) A suitable test should be devised for the early stage [9]. It has been shown that the onset of type 2 diabetes can be prevented or delayed by a combination of change in diet, moderate exercise and modest weight loss [2]. Early detection and intervention may already have an effect on reducing prediabetes and its adverse effect on optimal physiological function.

Biomarkers have played an important role in identifying and characterizing pathological processes with blood glucose levels (BGL), HbA1c and cholesterol levels having been measured as part of clinical assessment of diabetes and cardiovascular disease for many years [10]. The role of biomarkers is increasing as technical advancements allow more accurate investigation of biochemical processes involved in disease progression. Inflammatory markers such as C-reactive protein (CRP) and interleukins (IL’s) have been shown to predict development of type 2 diabetes [11] and are now used routinely in medical practice to screen for cardiovascular disease [12,13].

Diabetes progression is, however, not only connected with changes in traditional biomarkers such as BGL, HbA1c and cholesterol, but also inflammatory, endothelial dysfunction, oxidative stress and autonomic nervous system markers have been reported to be elevated in diabetes [12,14–20]. These include tumor necrosis factor α (TNFα), antioxidants such as reduced glutathione (GSH) and vitamin E (α-tocopherol), lipid peroxidation products such as malondialdehyde (MDA) and isoprostanes as well as markers of endothelial dysfunction including 8-hydroxy-2-deoxy-guanosine (8-OHdG) and homocysteine (Hcy) [12,14–20].

Current therapeutic interventions aim to reach certain target levels deemed to be appropriate to maintain health or reduce risk of disease progression. Statin use has been shown to be effective as it reduces LDL and triglycerides and increases HDL [21,22]. In addition, there is evidence that statins have antioxidant activity [23], but they do not necessarily lead to an improved CVD risk [24]. The association between oxidative stress and cardiovascular disease in diabetes via endothelial dysfunction and the resulting pathological changes in inflammation, coagulation status and cell-proliferation has been extensively investigated [18,25–27]. In the case of prediabetes, the situation is less understood [28,29], but changes in redox-status and the onset of hyperglycaemia-associated complications are already identifiable before diabetes diagnosis [20]. The central mechanism that is responsible for the increased cardiovascular risk in prediabetes is endothelial dysfunction due to the elevated formation of reactive oxygen species (ROS) and advanced glycation end products (AGEs) as well as increased lipid peroxidation under hyperglycaemic conditions [30–34]. The consequences of endothelial dysfunction include impaired balance between coagulation and fibrinolysis, platelet activation, vascular smooth muscle cell proliferation and stimulation of inflammatory processes, which all together create a pro-thrombotic environment and, if hyperglycaemia remains untreated, cause cardiovascular disease in the long-term [32,33].

The association between biomarkers and disease progression from normal to impaired fasting blood glucose (prediabetes) has not been studied extensively. Al-Aubaidy and Jelinek have recently shown that 8-OHdG is elevated already in the prediabetic state, suggesting an impact on pathological processes of even moderate increases in BGL [29]. If reliable and specific biomarkers for the prediabetic state can be established, early detection and treatment of prediabetes could slow down the worldwide diabetes epidemic.

This study aims to increase the understanding of the connection between oxidative stress and inflammation in prediabetes disease progression. The results of this study aim to provide a basis for improved diagnostic possibilities for detecting changes in prediabetes earlier, improved treatment and prevention in regular population screenings of people at increased risk of developing diabetes and cardiovascular disease. To achieve this, differences in the level of biochemical markers of oxidative stress (erythrocyte GSH/GSSG and urinary 8-isoprostane), inflammation (CRP, IL-6), endothelial dysfunction (homocysteine, urinary 8-hydroxy-2-deoxy-guanosine) and coagulation/fibrinolysis (CSA, D-Dimer) were determined in prediabetes and control subjects. To our knowledge, there has been no study so far that has investigated these markers together in prediabetes.

Material and methods

The study was approved by the Ethics in the Human Research Committee of Charles Sturt University.

Recruitment of participants

A total of 428 participants (female: male, 247:181) were selected within a rural diabetes screening clinic at CSU for analysis of blood and urine samples. There were no exclusion criteria for participation in this study except for age (participants older than 40 years were included). As part of the screening process BMI, gender, lifestyle profile including triglycerides (TG), HDL-cholesterol, LDL-cholesterol, TC/HDL ratio, and the atherogenic index of plasma (AIP) were determined for all participants.

The participants were divided into two groups: a control group (FBG b 5.6 mmol/L (100 mg/dL)) and a prediabetes group (FBG 5.6–6.9 mmol/ L (100–125 mg/dL)). Fasting blood glucose levels (FBG) for the two groups were defined according to the definition criteria of the American Diabetes Association 2004 [3].

Chemicals

The reagents used in this study were purchased from Cayman Chemical Company (Ann Arbor, MI, USA) (Interleukin-6 (human) ELA Kit Lot No. 0433189, Glutathione EIA Kit Lot No. 0440001 and 8-hydroxy-2-deoxy Guanosine EIA Kit Lot No. 0439545), BD Biosciences (San Jose, CA, USA) (Human C5a ELISA Kit II Lot No. 2146942) and Northwest Life Science Specialties (Vancouver, WA Canada) (Urinary Isoprostane ELISA Kit Lot No. 15002–1861).

Apparatus

Fasting blood glucose levels were determined using the Accu-Chek® system (Roche Australia Pty Ltd) and plasma homocysteine levels were measured with the automated AsYM® system (Abbott Laboratories, USA). All centrifugation procedures for the blood preparation were performed with a UNIVERSAL 32R (Hettich Zentrifugen, Germany). The photometric measurements to determine the levels of biomarkers in blood and urine were carried out with a Thermo Scientific Multiskan FC (Fisher, China).

Sample preparation

Venous blood was collected into EDTA-tubes to avoid coagulation, serum-separating-tubes (SST) and sodium-citrate (SC)-tubes to measure coagulation. The EDTA-treated samples were immediately separated into plasma and red cells by centrifugation for 15 min at 800 g and 4°C. The plasma was used to determine CSA, IL-6 and homocysteine levels. For measurement of erythrocyte reduced and oxidized glutathione (GSH and GSSG, respectively) an erythrocyte lysate was prepared by adding
4× volume of ice-cold 5% metaphosphoric acid to the red cells and centrifuging again for 10 min at 3000 g and 4 °C for deproteination. Urine was collected midflow for determination of 8-isoprostane and 8-OHdG.

Urine, plasma and erythrocyte lysate aliquots were either analyzed directly or stored at −80 °C until analysis. Additional urine aliquots, EDTA-, SST- and SC-tubes were sent to Dorevitch Pathology Laboratory, Albury, NSW, who provided urinary creatinine values, triglycerides, the cholesterol profile, HbA1c, CRP and D-Dimer levels.

**Measurement of oxidative stress**

Erythrocyte GSH and GSSG were determined by the Glutathione EIA Kit (Cayman Chemical, USA), which utilizes the enzyme glutathione reductase to measure total GSH. The 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) reaction produces the yellow colored 5-thio-2-nitrobenzoic acid (TNB), which can be measured spectrophotometrically at 405–414 nm and is directly proportional to total GSH concentration in the sample. For exclusive measurement of GSSG, the samples and standards were incubated with 1% of 2 M 2-vinylpyridine for 60 min at room temperature to derivatize free GSH before performing the assay. The concentrations of GSH and GSSG were determined using the End Point Method and corrected for the deproteination step [35,36].

8-isoprostane was determined by a urinary Isoprostane ELISA Kit (Northwest, USA), which uses a competitive ELISA strategy, allowing the 8-isoprostane contained in samples and standards to compete with a 8-isoprostane-horseradish peroxidase conjugate for binding to a specific antibody pre-coated on a microplate. The blue color development after addition of the horseradish peroxidase substrate is inversely proportional to the amount of 8-isoprostane in the samples and standards and changes to yellow after stopping the reaction with acid. Absorbance is measured at 450 nm.

**Measurement of endothelial dysfunction**

Urine 8-OHdG was determined by a 8-hydroxy-2-deoxy Guanosine EIA Kit (Cayman Chemical, USA), which is a competitive assay, using an anti-mouse IgG-coated plate and a tracer consisting of an 8-OHdG-acylcholinesterase conjugate instead of an antigen-coated plate to improve sensitivity and reduce variability. 8-OHdG in the sample competes with the 8-OHdG-enzyme conjugate (tracer) for a constant amount of 8-OHdG monoclonal antibody. The tracer-antibody complex binds to the pre-coated anti-mouse IgG and addition of AChE-substrate (acylcholine and 5,5′-dithio-bis-2-nitrobenzoic acid) results in the enzymatic production of a yellow 5-thio-2-nitrobenzoic acid, which can be measured spectrophotometrically at 412 nm and is inversely proportional to the amount of 8-OHdG in the original sample. Quantification of creatinine in the urine samples was done by Dorevitch Pathology Laboratory, Albury, NSW to normalize both 8-isoprostane and 8-OHdG values.

Plasma homocysteine was determined by the automated AxSYM® system (Abbott Laboratories, USA), which uses a two-step reaction (1. reduction of protein-bound homocysteine by dithiothreitol, 2. enzymatic conversion of free homocysteine to S-adenosylhomocysteine in the presence of adenosine). The product of reaction 2 (S-adenosylhomocysteine) is detected by a fluorescence polarization immunoassay.

**Measurement of inflammation**

Plasma IL-6 was determined by an Interleukin-6 (human) EIA Kit (Cayman Chemical, USA), which uses a double-antibody sandwich ELISA strategy. Free IL-6 in the samples binds to the pre-coated monoclonal antibody specific for IL-6 and an acetylcholinesterase:IL-6 Fab’ conjugate binds selectively to a different epitope on the IL-6 molecule. Addition of AChE-substrate (acylcholine and 5,5′-dithio-bis-2-nitrobenzoic acid) results in the enzymatic production of the yellow 5-thio-2-nitrobenzoic acid, which absorbs at 412 nm. The color development is directly proportional to the concentration of IL-6 in the sample.

Serum CRP levels were provided by Dorevitch Pathology Laboratory, Albury, NSW.

**Measurement coagulation and fibrinolysis**

Plasma C5a was determined by the Human C5a ELISA Kit II (BD Biosciences, USA), which is a sandwich ELISA assay using monoclonal antibody specific for human C5a pre-coated on microplates. The second antibody, which binds to immobilized C5a, is an anti-human C5a antibody — streptavidin horseradish peroxidase conjugate, which reacts with a peroxidase substrate (TMB) to a blue product. The reaction is stopped by addition of phosphoric acid, which changes the color to yellow. Absorbance can be measured at 450 nm and is directly proportional to the amount of C5a in the initial sample.

D-Dimer levels were provided by Dorevitch Pathology Laboratory, Albury, NSW.

**Statistical analysis**

Sample concentrations were calculated using Microsoft Excel (Office2007, Microsoft) and descriptive data is expressed as mean ± standard deviation (x ± SD). Statistical analysis was performed with SPSS (Version 20, IBM Co). To determine if there were significant differences in biomarker levels between the control and prediabetes group a Student t-test was used. A p-value <0.05 was considered as significant.

**Results**

Table 1 shows the main characteristics and biomarker results of the two groups. The control and prediabetic subjects were comparable concerning age (61.81 ± 11.58 and 66.99 ± 10.82), distribution of gender (slightly higher proportion of females: 59.9% and 54.6%) and BMI (26.29 ± 4.64 and 28.22 ± 5.12, p > 0.05). The prediabetes group had
about 3 times more smokers (10.8%) than the control group (3.97%) and some minor differences were present in the medication status. While the percentage of subjects on antihypertensives was remarkably higher in the prediabetes group (35.8%) compared to the control group (5.2%), the number of subjects on statins and anticoagulants (aspirin/clopidogrel) was relatively small in both control (<4%) and prediabetes group (<16%). The blood lipid levels showed only minimal differences between both groups, being slightly improved in the prediabetes group with significant lower total cholesterol (p = 0.02) and HDL (p = 0.004) in the latter.

Reduced glutathione levels were slightly increased in the prediabetes group, while the increase in oxidized glutathione (GSSG) levels compared to control was a bit more prominent. Neither of them showed significant changes. However, the GSH/GSSG ratio was significantly (p = 0.001) decreased in the prediabetes group. No difference was found in the 8-isoprostane levels between both groups (control: 0.21 ± 0.16, prediabetes: 0.23 ± 0.19 mg/mmol creatinine).

Both urinary 8-OHdG and plasma homocysteine levels were significantly higher (p = 0.04 and p = 0.01, respectively) in the prediabetes group compared to control.

We also observed increased inflammation in the prediabetes group, represented by elevated CRP (control: 3.05 ± 2.30, prediabetes: 3.69 ± 2.72 mmol/L) and IL-6 levels (control: 28.66 ± 28.43, prediabetes: 34.37 ± 33.72 pg/mL) in the prediabetes group compared to control, both with a p-value <0.1.

The same trend was found for the coagulation/fibrinolysis status. Both C5a (control: 19.32 ± 20.04, prediabetes: 21.91 ± 21.03 ng/mL) and D-Dimer (control: 0.46 ± 0.30, prediabetes: 0.54 ± 0.37 μg/L) levels were increased in the prediabetic subjects, but the differences did not reach significance (p > 0.05).

Discussion

Prediabetes is the asymptomatic stage of diabetes mellitus and might be a useful target for early intervention therapies to prevent the development of this wide-spread disease and its associated complications like cardiovascular disease. We focused on changes in biomarkers, which can already be seen in the prediabetic state, to provide new knowledge about disease progression and possible tools for prevention screening programs. Based on the definition of prediabetes in this study as elevated fasting blood glucose levels between 5.6 and 6.9 mmol/L we expected to find adverse effects of mild hyperglycaemia manifested in impaired biomarker levels in the prediabetes group.

Unexpectedly, the prediabetic subjects were characterized by a slightly improved blood lipid profile. A possible explanation for that phenomenon is that even though the differences in statin use between the two groups were only small, this could still have had an influence on the blood lipid outcomes of the prediabetes group, since statins lower LDL and increase HDL levels.

A very important finding was the significantly decreased GSH/GSSG ratio in the prediabetes group, which indicates an impaired redox status in the prediabetes group compared to the controls. This result proves the presence of increased oxidative stress already at the prediabetes state, which is represented by a slight increase in GSSG levels together with a significant decrease in GSH/GSSG ratio in the prediabetes group. This is consistent with earlier findings, which showed that changes in the antioxidant status, especially the glutathione system of the erythrocytes, characterize the initial phase of oxidative stress in diabetes mellitus progression and commence prior to the establishment of this disease [28]. The minimal increase in GSH levels in the prediabetes group could be due to the antioxidant effect of hypoglycaemic and cardiovascular disease medication. Another possible explanation is an activation of GSH synthesis in response to increased GSSG formation due to increased oxidative stress, probably caused by higher amounts of ROS in the erythrocytes. Dincer et al. proved that oxidative stress inhibits the activity of glutathione reductase, the enzyme that regenerates GSH from GSSG [18], an effect, which could also contribute to the increased GSSG levels and new synthesis of GSH. As a second marker for oxidative stress we chose 8-isoprostane, because it is said to be a reliable indicator of oxidative stress and lipid peroxidation in humans [37]. Physiologically, 8-isoprostane is generated through the non-enzymatic oxidation of phospholipids containing arachidonic acid and then excreted into the urine [31]. Our study did not find any changes in urinary 8-isoprostane levels from control to prediabetic subjects, indicating that the antioxidant system of the prediabetic subjects, including the GSH/GSSG cycling, was still capable of preventing further oxidative damage to lipids. Therefore we conclude, that urinary 8-isoprostane is not sensitive enough to be a useful biomarker in the screening for prediabetes.

The strongest evidence for altered functional status at the prediabetic state compared to control were the significant changes in endothelial dysfunction related to levels of 8-OHdG. 8-OHdG is a product of DNA base modification at the C-8 site produced by oxidation of deoxyguanosine [29]. It is the most sensitive marker of oxidative DNA damage and moreover reflects total systemic oxidative stress in vivo [20,38]. In addition, 8-OHdG is an established risk marker for atherosclerosis and diabetes mellitus and it has been shown that urinary 8-OHdG is more stable than serum 8-OHdG [39], which is why we chose to use urine samples for the measurement of this marker. Homocysteine is a cytotoxic amino acid, which is secreted into the plasma from endothelial and red blood cells as a product of incomplete conversion of methionine to cysteine, reflecting increased oxidative stress [40]. Studies on the connection between plasma homocysteine level and CVD risk have been controversial, with some authors claiming an association [41] and some denying the efficacy of lowering the homocysteine levels as a means of preventing the CVD incidence [42]. There is however consensus about the prothrombotic and prooxidant properties of homocysteine, which can cause the formation of ROS and contribute to endothelial dysfunction [40–42]. Homocysteine can moreover inhibit the expression of antioxidant enzymes like glutathione peroxidase-1, which has the consequence that the regeneration of reduced glutathione is limited [41]. In this study we found not only significantly higher 8-OHdG (p = 0.04), but surprisingly also significantly increased homocysteine levels (p = 0.009) in the prediabetic subjects compared to control. The elevated 8-OHdG level together with the impaired GSH/GSSG ratio is consistent with earlier findings, where serum 8-OHdG and glutathione status have been measured [29,38]. Urinary 8-OHdG can be measured non-invasively and the samples are easier to handle compared to blood, which makes it a better alternative to the traditional serum 8-OHdG, if it shows the same sensitivity. We have shown that the measurement of urinary 8-OHdG gives the same results for prediabetic patients as the measurement of serum 8-OHdG. The elevated homocysteine was unexpected, but shows once more that increased oxidative stress is accompanied with changes in intracellular metabolism, as in the case of cysteine production, and endothelial dysfunction, reflected in the elevated plasma homocysteine levels. However, it is also possible that homocysteine contributes itself to the oxidative stress by stimulation the formation of ROS. Either way, the association is apparent and homocysteine seems to be elevated already in the prediabetic state. These results prove that the relatively small elevation of blood glucose levels in the prediabetic state has a detectable influence on endothelial function, resulting in increased DNA-damage and homocysteine release from endothelial cells. Increased oxidative stress as measured by the impaired GSH/GSSG ratio is likely to be the link between the moderate hyperglycaemia in prediabetes and pathological changes in endothelial function, which in the long-term may promote atherogenesis and result in the development of cardiovascular disease. The observed non-significant but consistent trend of increased inflammation and coagulation in prediabetes measured by slightly elevated CRP, IL-6, C5a and D-Dimer reflects the pathological consequences of hyperglycaemia-induced oxidative stress in prediabetes and underlines the findings that have been discussed above. CRP is the golden marker of inflammation and is said to be a better predictor of CVD risk than LDL [43] and elevated levels of both CRP and IL-6 predict the
development of type 2 diabetes mellitus [11]. Our findings confirm that, since the prediabetic subjects showed higher levels of both markers compared to control, which shows the importance of assessing markers of inflammation in screening for prediabetes, early detection of the preclinical state is essential. C5a is a potent proinflammatory complement component, which among others induces adhesion molecule expression and tissue factor activity in endothelial cells. It is therefore involved in coagulation pathways [44] D-Dimer is an indicator of coagulation activity, since it is the product of the enzymatic degradation of fibrin, a process called fibrinolysis [45]. Both increased and decreased levels have so far been reported in diabetes mellitus patients [46]. Our results showed that there is a trend towards elevated C5a and D-Dimer levels in the prediabetic subjects, which indicates that there probably is a slightly increased coagulation activity already in the prediabetic state, leading the way for future cardiovascular complications.

To our knowledge, this study was the first one that examined changes in oxidative stress, endothelial dysfunction, inflammation and coagulation/fibrinolysis together in a prediabetes group compared to controls. The results show that the onset of endothelial dysfunction and increased oxidative stress manifest early in disease progression and are detectable as changes in biomarker levels are already at the prediabetic state. Also the tendency towards increased inflammation and coagulation was noticeable in the prediabetic subjects, being well-known risk factors for the development of macrovascular complications. These findings not only prove once more, that prediabetes is a clinical state that needs to be taken seriously since it is the precursor of diabetes, but much more importantly offer reliable tools for early detection of diabetes and the associated complications like cardiovascular disease in prevention screenings.

Acknowledgments

We gratefully thank Amanda Eddy for her technical assistance. Laura Maschiuro received a travel scholarship from the German Academic Exchange Service (DAAD).

References

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