

Full Length Research Paper

Diabetes, oxidative stress and cardiovascular risk

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ABSTRACT

Type 2 Diabetes Mellitus (T2DM) and associated cardiovascular disease (CVD) is approaching global epidemic proportions with no signs of abatement. This current study examined correlations between inflammation and oxidative stress in (T2DM) and the Framingham CVD risk score. A cross sectional cohort of patients enrolling in the Diabetic Complications Research Initiative at Charles Sturt University was examined for diabetes status and divided into control, prediabetic, and a T2DM groups. The cohort was also divided with respect to Framingham CVD risk categories of low, moderate and high risk. Fasting lipid levels, blood glucose, glycated haemoglobin (HbA1c), interleukin 6, (IL-6), glutathione (GSH) and glutathione disulfide (GSSG) were measured. Body Mass Index (BMI), blood pressure and estimated glomerular filtration rate (eGFR) were included. Significant correlations in diabetes status and CVD risk with GSH and IL-6 were observed. This study further supports previous data that inflammatory processes and oxidative stress are implicated in T2DM and CVD risk.

Keywords: Type 2 Diabetes Mellitus, Prediabetes, Cardiovascular disease, Oxidative stress, Risk factors, Body Mass Index, Glutathione, Glutathione disulfide, Interleukin-6

INTRODUCTION

Approximately, 382 million or 8.3% of the world population are known to have diabetes mellitus (DM). This number may rise beyond 592 million in less than 25 years (Federation, 2013). However approximately 30% of people remain undiagnosed for substantial time (Gholap et al., 2013). As a consequence diabetic complications including eye, heart and kidney disease are often undiagnosed until they are in an advanced state, requiring more intensive medical intervention.

Increased risk of complications are already associated with the prediabetes state and associated with oxidative stress mechanisms (Yan et al., 2003). The prediabetic state is defined as an impaired fasting glucose (IFG) level

higher than the normal glucose reference range but below that diagnostic for diabetes (American Diabetes Association, 2004). The American Diabetic Association classify the prediabetic state as an IFG of equal or greater than 5.6 mmol/l but less than 7 mmol/l. Any rise in IFG may lead to the development of diabetes associated complications caused by the increased blood sugar level and ensuing oxidative stress (Giacco and Brownlee, 2010; Tiwari et al., 2013)

Many studies and reviews have been conducted to assist in clarifying the role oxidants play in the progression of CVD as a complication in diabetes (Schrijvers et al., 2007). As early as the mid nineteenth

century Virchow's concept of the atheroma as resulting from injury, inflammation and the immune response supported this view and pointed to multiple independent pathways contributing to atherosclerotic risk and cardiovascular morbidity and mortality (Libby et al., 2009).

Oxidative stress

Oxidative stress (OS) is defined as an imbalance of free radical production and the associated antioxidant defence mechanisms (Stocker and Keaney, 2004). Persisting hyperglycaemia evident in T2DM, initiates OS by an increase in both intercellular and extracellular free radical levels in the blood (Al-Aubaidy and Jelinek, 2011; Whiting et al., 2008). The most ubiquitous pool of antioxidants is erythrocyte reduced glutathione (GSH), which responds to excessive free radicals. Free radicals and reactive oxygen species (ROS) entering the blood stream are detoxified by the antioxidant activity of GSH (Al-Aubaidy and Jelinek, 2010; Ballatori et al., 2009). GSH is known to act as an electron donor participating in the conjugation reaction of Glutathione-S-transferase for detoxifying endogenous compounds. In addition GSH aids in the reduction of methaemoglobin to haemoglobin; and the regeneration of antioxidant vitamins such as Vitamin C. GSH is a known substrate for Glutathione peroxidase 1 (Gpx1) with the selenium dependent form of Gpx1 acting in association with GSH catalysing peroxides resulting in the oxidation of GSH (Rahman, 2007). An increased activity of Gpx1 associated with a decreased GSH activity can result in the increased production of Glutathione disulfide (GSSG), the oxidized form of GSH (Ahmed, 2005). Thus the ratio of GSH to GSSG can be utilised as a useful marker in the assessment of the antioxidant status (Jelinek et al., 2014). Depleted GSH levels as occur in the case of chronic hyperglycaemia due to the loss of cysteine and cysteine transport mechanisms across the erythrocyte membrane leads to a decrease in many cellular antioxidant defence pathways (Bannai and Tateishi, 1986; Toroser and Sohal, 2007) and increased risk of CVD morbidity and mortality. Normally erythrocytes have a flexible membrane that allows membrane channels to transport GSH precursors such as cysteine. However, erythrocyte oxidative stress (EOS) contributes to cell membrane inflexibility reducing cross-membrane transport. The increase in erythrocyte rigidity also leads to an increase in blood viscosity, which is a marker for increased risk of CVD (Irace et al., 2014).

Interleukin-6, inflammatory response in T2DM and CVD

Atherosclerosis is predominantly the result of an inflammatory process driven by proinflammatory

cytokines. High levels of the proinflammatory interleukin-6 (IL-6) have been associated with obesity and insulin resistance and a contributory role in the pathogenicity of diabetes, CVD and coronary heart disease (CHD) (Kristiansen and Mandrup-Poulsen, 2005; Lowe et al., 2014; Yudkin et al., 2000). Inflammatory processes and oxidative stress in T2DM may be intricately connected as multiple regression analysis of lipid peroxidase and IL-6 was found to correlate independently with C reactive protein (CRP) (Arnalich et al., 2000).

Cardiovascular diseases in diabetes mellitus

Studies have shown that diabetes itself and various diabetes associated progressive biochemical reactions such as oxidative stress, pro-coagulation activity and inflammation can generate atherosclerosis (Al-Aubaidy and Jelinek, 2014) and have a higher risk of death as a consequence of cardiovascular disease (CVD) when compared to patients with prior evidence of CVD but without diabetes (Juutilainen et al., 2005). The Framingham risk equation for CVD considers age, gender, blood pressure, diabetes status and cholesterol levels as factors in 5-year risk of CVD (Abraham et al., 2015; Perreault et al., 2014).

The current study aimed at determining the role of inflammatory cytokines and oxidative stress in diabetes disease status and their association with the Framingham CVD risk.

MATERIAL AND METHODS

The study protocol was reviewed and approved by the ethics in human research committee of Charles Sturt University in accordance with the provisions set out in the Declaration of Helsinki. Informed consent was obtained from each participant after a full explanation of the purpose, nature, and risk of all the procedures used was provided by the principal investigator. Samples of blood and urine were collected from 60 participants. The body mass index (BMI), blood pressure, waist circumference, age and gender were obtained. All participants were classified according to their fasting blood glucose levels (BGL). A control group was defined as a fasting BGL of <5.6mmol/L, a prediabetic group with a fasting BGL of 5.6 – <7mmol/L and a T2DM group according to a fasting BGL of \geq 7.0mmol/L and no recorded comorbidity (American Diabetes Association, 2004).

Participants were also sub-divided into three CVD risk groups as suggested by the Australian Heart Foundation Risk levels based on the Framingham 10 year risk categories as low, moderate high risk for CVD (Davis et al., 2010). Lipid profiles for total cholesterol (TC), Triglycerides (TG), HDL-Cholesterol, LDL-Cholesterol and the TC/HDL ratio were also determined. Blood

glucose levels (BGL), glycated haemoglobin (HbA1c), blood pressure, waist circumference, body mass index (BMI) and glomerular filtration rate were also measured.

Preparation of samples

After an overnight fast, whole blood specimens were collected into 9 mL heparin and ethylene diamine tetra acetic acid (EDTA) tubes for analysis. Heparinised plasma was separated within 1 hour by centrifugation at 1000 g for 10 minutes. Blood glucose level (BGL), glycosylated haemoglobin (HbA1c), Total Cholesterol (TC), High density lipoprotein (HDL-C) and triglycerides (TG) were analysed at the local pathology laboratory in accordance with Australian Laboratory Standards. Fasting plasma TC and TGs were determined with a commercial enzymatic kit. High-density lipoprotein cholesterol (HDL-C) was determined by immunoinhibition assay and LDL-C was calculated according to the Friedewald formula (Friedewald et al., 1972).

The EDTA specimen was used to prepare the plasma, buffy coat, washed red cells and erythrocyte lysate. Specimens were centrifuged at 800 g for 15 minutes at 4 °C and the plasma and the buffy coat layer were transferred to separate tubes for testing. To obtain washed red cells the cells were suspended and washed four times in Dulbecco's Phosphate Buffered saline. The preparation of the red cell lysate involved diluting whole cells suspended in 4 X volume of metaphosphoric acid vortexed and re-centrifuged. A urine specimen was also collected from each client.

Measurement of oxidative stress

GSH and GSSG levels were measured from erythrocyte lysate by a Glutathione Assay Kit (Cayman Chemical, USA, Lot No. 0428439 and 0431962). As the method incorporates glutathione reductase, total glutathione is measured. GSH reacts with Ellman's reagent with the production of 5-thio-2nitrobenzoic acid (TNB); optical density was measured at 405nm with the absorbance of TNB directly proportional to the GSH concentration in the sample. Results were calculated using a four parameter logistic fit.

Measurement of IL-6

IL-6 levels were determined using the human IL-6 Enzyme Linked Immunosorbent Assay (ELISA) kit (Elisakit.com, Adelaide Australia). The method incorporated pre-coated IL-6 capture antibody incubated with anti-human IL-6 biotin labelled detection antibody.

Plates were developed with Streptavidin – horse radish peroxidase (HRP) conjugate. Colour development

occurred utilising 3,3',5,5'-tetramethylbenzidine TMB chromogen and was stopped with kit supplied acid solution. The absorbance of the resultant yellow colour was determined at 450nm. Results were calculated by generating a standard curve using a four parameter logistic fit. All ELISA assays were measured by a Thermo Scientific Multiskan FC and data reduction utilised SkanIt 3.1 software.

Statistical analysis

The statistical analysis was performed using Microsoft Excel (Office 2007, Microsoft) and PAWS Statistics 22 (IBM). All data are described as mean ± S.D. The significance was tested using ANOVA followed by Fischer's Least Square Difference post hoc test for group comparisons.

RESULTS

Table 1 shows the main characteristics of the subjects included in this study.

Significant differences were identified in the ANOVA for age, SBP, BGL, HbA1c, LDL, IL-6 and GSH/GSSG. Fischer's LSD post hoc analysis indicated a significant difference between control and the prediabetic group for BGL and GSH/GSSG with BGL increasing and GSH/GSSG decreasing. In T2DM IL-6 was also significantly higher compared to control in addition to age, SBP, BGL, HbA1c, which were all significantly higher. IL-6 level further increased significantly in the T2DM group compared to the prediabetes group. LDL was significantly lower in the prediabetes and T2DM groups most likely due to medication use. SBP was significantly higher in the T2DM group compared to control and GSH/GSSG further decreased but was not significant ($p < 0.058$).

One-way ANOVA results for CVD risk subgroup analysis indicated significant differences for age, BGL, SBP, HbA1c, Total Cholesterol, LDL, IL-6 and GSH. Fischer's LSD post hoc analysis showed significant increases in BGL, SBP, total cholesterol, LDL and IL-6 between the low and moderate CVD risk groups. BGL and HbA1c were significantly higher in the high CVD risk group compared to the moderate CVD risk group, whereas GSH was significantly lower. Age, SBP, BGL, HbA1c, total cholesterol and LDL were significantly higher in the high CVD risk group compared to the low CVD risk group. BGL and HbA1c increased significantly from the moderate to the high CVD risk group and GSH decreased significantly. Triglycerides trended upwards with HDL decreasing but not significantly. Similarly eGFR, whilst not significant, did show a downwards trend with increasing CVD risk categories. IL-6 was still higher in the high CVD risk group compared to control but was lower compared to the moderate CVD risk group.

Table 1. Clinical biomarkers for control, prediabetic and T2DM groups

	Controls	Prediabetes	T2DM
Age (years)	62.02 ± 9.93	66.80 ± 10.98	73.25 ± 11.18 ¹
Sex (Female/Male)	21/15 (n=46)	9/6 (n=20)	3/6 (n=9)
BGL (mmol/L)	5.12 ± 0.38	6.09 ± 0.37 ²	7.74 ± 2.59 ^{1,3}
HbA1c (%)	5.62 ± 0.44	5.71 ± 0.39	7.00 ± 0.1.10 ^{1,3}
Smoking (% yes)	0	13.33 %	0
Alcohol (% yes)	2.78 %	6.67 %	33.33 %
SBP _{av} (mmHg)	119 ± 12	122 ± 13	129 ± 11 ⁴
DBP _{av} (mmHg)	73 ± 7	74 ± 7	74 ± 7
eGFR (mL/min/1.73m ²)	92.45 ± 17.03	90.78 ± 22.42	90.95 ± 18.69
Total cholesterol (mmol/L)	4.97 ± 1.92	5.08 ± 1.57	3.76 ± 0.1.67
Triglycerides (mmol/L)	1.00 ± 0.67	1.02 ± 0.62	1.20 ± 0.65
HDL (mmol/L)	1.69 ± 0.36	1.66 ± 0.45	1.75 ± 0.76
LDL (mmol/L)	3.39 ± 0.82	3.19 ± 0.82	2.02 ± 0.87 ^{1,3}
IL-6 (pg/mL)	23.43 ± 16.34	20.28 ± 14.47	41.79 ± 40.07 ^{4,6}
GSH (μmol/L))	1632.48 ± 613.87	1515.85 ± 592.83	1337.51 ± 606.81
GSSG (μmol/L)	357.62 ± 244.86	416.16 ± 177.32	375.08 ± 188.99
GSH/GSSG	7.80 ± 6.09	4.20 ± 2.08 ⁵	4.04 ± 2.44

¹ Significant difference between control and T2DM (p < 0.01).

² Significant difference between control and prediabetes (p < 0.01).

³ Significant difference between prediabetes and T2DM (p < 0.01)

⁴ Significant difference between control and T2DM (p < 0.05)

⁵ Significant difference between control and prediabetes (p < 0.05)

⁶ Significant difference between prediabetes and T2DM (p < 0.05).

BMI – body mass index, eGFR - estimated glomerular filtration rate, HDL – high density lipoprotein, LDL – low density lipoprotein, CVD risk – cardiovascular disease risk, IL-6 Interleukin 6, GSH – glutathione, GSSG – glutathione disulfide, GSH/GSSG ratio, SBP – Systolic blood pressure, DBP, Diastolic blood pressure.

DISCUSSION

This study highlights the importance of measuring emerging biomarkers of oxidative stress and inflammatory markers as indicators of T2DM progression and association with CVD risk. The inflammatory marker, IL-6 and the emerging oxidative stress markers including GSH and GSH/GSSG were significantly different between subgroups when diabetes progression or when the Framingham CVD risk increase is investigated. GSH has previously been shown to be useful for elucidating associations between impaired fasting glucose and oxidative stress in diabetes and CVD progression (Al-Aubaidy and Jelinek, 2010; Lowe et al., 2014; Nwose et al., 2006; Nwose et al., 2008).

GSH levels were significantly higher in the moderate CVD risk group compared to the low CVD risk group and were significantly decreased in the high CVD risk group compared to the moderate CVD risk group (Table 2). This increase and then decrease in GSH was not observed within the diabetic subgroup results. In fact the trend was opposite with GSH levels being lower in the prediabetic group compared to control and then increasing again in the T2DM group. This suggests that the mechanisms associated with the changes in antioxidant activity of GSH may be quite different with diabetes progression and oxidised GSH forming GSSG. GSSG did increase in both the prediabetic and moderate CVD risk groups and

returned to near control levels indicating a cyclic change in the redox state. Interestingly the GSH/GSSG provided significant results in the diabetes subgroup analysis providing further confirmation of previous findings that GSH/GSSG may be a more sensitive marker compared to GSH, possibly due to GSH being present in much larger quantities in the blood. This indicates an early thiol related oxidative stress response and impaired redox state of erythrocyte GSH (Jelinek et al., 2014; Kadiska et al., 2001; Maschirow et al., 2015). Previously Al-Aubaidy and Jelinek (2010) reported that GSH levels were significantly decreased in a non-medicated prediabetic group possibly indicating an early state of oxidative stress and the current nonsignificant difference may be due to lower patient numbers or a slightly different cholesterol or inflammatory profile. In addition normal levels of GSH were observed in a group of patients reporting T2DM for 2 years suggesting that either better medical treatment or lifestyle practices allowed GSH to return to normal levels. Our hypothesis based on a previous study is that the erythrocyte GSH pool decreases at first with increased BGL but due to any de novo synthesis in response to continued elevated BGL, GSH rebounds and increases, whilst erythrocytes remain functional (Al-Aubaidy and Jelinek, 2011; Jelinek et al., 2014; Nwose et al., 2006; Nwose, Jelinek et al., 2007; Nwose et al., 2008).

The increased erythrocyte generated ROS observed with diabetes progression and CVD risk enhances

Table 2. CVD risk groups

	Low risk	Moderate risk	High risk
Age (years)	62.2 ± 9.66	77.0 ± 7.0 ⁵	81.33 ± 11.59 ¹
BGL (mmol/L)	5.40 ± 0.62	6.29 ± 1.71 ⁵	9.45 ± 1.77 ^{1,3}
HbA1c (%)	5.58 ± 0.42	6.03 ± 0.86 ⁵	7.85 ± 0.07 ^{1,3}
Waist circumference (cm)	89.13 ± 10.58	94.44 ± 12.59	99.00 ± 8.54
BMI (kg/m ²)	25.57 ± 4.53	25.11 ± 1.76	24.33 ± 0.58
SBP _{av} (mmHg)	118 ± 11	133 ± 12 ²	137 ± 12 ⁴
DBP _{av} (mmHg)	73 ± 6	74 ± 6	75 ± 3
eGFR (mL/min/1.73m ²)	94.09 ± 16.82	84.21 ± 26.08	78.30 ± 18.71
Total cholesterol (mmol/L)	5.62 ± 0.82	4.98 ± 1.00 ⁵	4.03 ± 0.91 ⁴
Triglycerides (mmol/L)	1.10 ± 0.60	1.20 ± 0.43	1.60 ± 0.87
HDL (mmol/L)	1.75 ± 0.33	1.72 ± 0.59	1.47 ± 0.76
LDL (mmol/L)	3.38 ± 0.78	2.70 ± 0.89 ⁵	1.77 ± 0.99 ¹
GSH (µmol/L)	1516.16 ± 622.31	1930.41 ± 559.94 ⁵	951.49 ± 579.33 ⁶
GSSG (µmol/L)	322.13 ± 187.00	405.09 ± 143.58	322.68 ± 207.57
GSH/GSSG	7.14 ± 5.86	5.40 ± 2.77	3.01 ± 0.16
IL-6 (pg/mL)	22.46 ± 14.40	38.07 ± 37.93 ⁵	29.58 ± 29.69

¹ Significant difference between low and high CVD risk (P < 0.01).

² Significant difference between low and moderate CVD risk (P < 0.01).

³ Significant difference between moderate and high CVD risk (P < 0.01)

⁴ Significant difference between low and high CVD risk (P < 0.05)

⁵ Significant difference between low and moderate CVD risk (P < 0.05)

⁶ Significant difference between moderate and high CVD risk (P < 0.05).

CVD – Cardiovascular, BMI – body mass index, SBP – Systolic blood pressure, DBP – Diastolic blood pressure, eGFR - estimated glomerular filtration rate, BGL – Blood glucose, HDL – high density lipoprotein, LDL – low density lipoprotein, HDL – high density lipoprotein, LDL – low density lipoprotein, GSH – glutathione, GSSG – glutathione disulfide, GSH/GSSG - ratio, IL-6 - Interleukin 6.

activation of the nuclear redox sensitive transcription factors resulting in the up regulation of genetic events such as procoagulant factors and proinflammatory mediators including IL-6, which participate in endothelial dysfunction and CVD (Nwose et al., 2007).

Diabetes progression showed a significant increase in IL-6 in the T2DM group when compared to the control group and the pre-DM group, whereas the CVD risk subgroup analysis showed a significant difference between the low and moderate risk group. High levels of IL-6 are associated with obesity and insulin resistance and play a role in the development of CVD (Dandona et al., 2004). The results from Dandona's study also provided evidence that IL-6 is associated with macrovascular complications in T2DM patients, however caution in interpreting or attempting to obtain any conclusive correlation between IL-6 in T2DM and CVD needs to consider that T2DM is a risk factor for CVD whilst IL-6 plays a role in the development of CVD in DM patients (Schöttker et al., 2013). Yudkin has proposed that IL-6 may contribute to atherothrombosis in coronary disease via a number of differing mechanisms including metabolic, endothelial and procoagulant effects (Yudkin et al., 2000). Supported by findings in this study and in agreement with Lowe et al (2014), further analysis in IL-6 to test the thesis that levels may independently assist in a better prediction of macrovascular events in patients with T2DM and CVD risk is required. Recognising the

incidence of CVD occurring in T2DM patients these results may also demonstrate, despite a commonality of risk factors, that there may be some independent pathogenesis occurring (Martín-Timón et al., 2014).

In the current study the more traditional risk factors including cholesterol profile were also investigated. The total cholesterol (TC) values of the diabetic group and CVD risk groups were lower than the levels of the prediabetic group and controls (Table 1 and 2). However we retained T2DM patients on medication and hence 56% of the T2DM patients were medicated with statins. Statins act by inhibiting hydroxyl methyl glutamyl Co-A (HMG-CoA) reductase, thus decreasing total and LDL cholesterol levels and reducing the risk of CVD (Ebrahim et al., 2014). As a corollary to lowering cholesterol levels the use of statins has also been shown to provide an improvement in eGFR in patients with diabetes, hypertension and glomerular nephritis (Sandhu et al., 2006). Estimated GFR in our study was normal in the T2DM group (Table 1) and in the high CVD risk group (Table 2) indicating a possible effect of medication. LDL levels in the T2DM did show a significant difference and improvement with decreasing levels possibly related to anti-diabetic and statin medication (Batsis and Lopez-Jimenez, 2010). Increased levels of TG and hyperglycaemia are considered risk factors for CVD, with evidence suggesting that both induce endothelial dysfunction through oxidative stress (Ceriello et al.,

2002). Triglyceride levels were not significant in this study. As a biomarker for CVD risk triglycerides have been debated for over three decades, however its known inverse association with HDL, the link with LDL, atherogenic cholesterol-enriched remnant lipoprotein (RLP's), CVD risk and T2DM makes its use as a biomarker essential (Miller et al., 2011). Triglyceride analysis in conjunction with oxidative stress, inflammatory, coagulative and fibrinolytic markers may provide additional insights into the development of CVD and T2DM. Increasing BGL with increasing CVD risk as expected is evident.

Long-term glucose dysfunction is associated with HbA1c a well-established indicator of glycaemia monitoring and risk screening for T2DM (Lerner et al., 2014). HbA1c levels were observed to be within normal limits in the prediabetic group (under 6 %). For the type 2 diabetes group, HbA1c levels were above 6%, which is slightly elevated, and supports extensive prior studies in poor glycaemic control such as the Diabetes Control and Complications Trial and Follow-up Study (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS) (Bennett et al., 2007). BGL, HbA1c and the oral glucose tolerance test are good screening tools for identifying and monitoring T2DM but have not been shown to have a high sensitivity for CVD progression (Cederberg et al., 2010; Chamnan et al., 2013; Pradhan et al., 2007; Tuomilehto, 2002). The findings in this study indicate that both fasting BGL and HbA1c are significantly associated with diabetes and a high CVD risk. However, BGL and HbA1c are not as useful for early preclinical assessment of prediabetes and moderate CVD risk (Dawber et al., 1951), unlike the "emerging markers" such as glutathione and IL-6.

Larger cohort studies for changes in IL-6 and GSH and GSSG in combination with traditional biomarkers and correlated to medication use are however required. Prediction of ensuing disease processes and progression through the use of anthropometry, other biological indices and biomarkers will aid in obtaining more conclusive evidence in the search for reliable markers which currently remain inconclusive

CONCLUSION

The study supports previous findings that inflammatory and oxidative stress provide useful information when considering the progression to T2DM and increased CVD risk. A novel finding was the significant results of the oxidative stress marker GSH/GSSG and the inflammatory marker IL-6 in the DM group. GSH was significant with respect to CVD risk status. This further highlights the importance that oxidative stress and inflammatory markers may contribute to our understanding of the processes involved in the development of T2DM and CVD progression.

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