Emerging Biomarkers of Oxidative Stress and Inflammation as Indicators of Progression to Type 2 Diabetes Mellitus and the Association with Cardiovascular Disease

Eugene G. Butkowski

BAppSc, Charles Sturt University, 1982
MAppSc, Charles Sturt University, 1994
LLB, Macquarie University, 2000
GDipLegPrac, Australian National University, 2001

A Thesis submitted to Charles Sturt University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

March 2016
# Contents

Certificate of Authorship................................................................. vii

Dedication ........................................................................................ vili

Acknowledgments............................................................................... ix

Ethics Statement................................................................................ xi

List of Abbreviations.......................................................................... xii

List of Tables...................................................................................... xiv

List of Figures..................................................................................... xv

Publications arising from doctoral research....................................... xvi

Publications listed in Appendices....................................................... xvii

Publications – Submitted final versions............................................. xvii

ABSTRACT......................................................................................... xviii

Chapter 1 .......................................................................................... 1

1. General overview of the issue:...................................................... 1

1.1 Atherosclerosis........................................................................... 5

1.2 Lipid metabolism and contribution to T2DM and CVD.............. 8

1.2.1 Lipid biochemistry................................................................... 8

1.2.2 Lipid classification................................................................. 9

1.2.2.1 Apolipoproteins............................................................... 12

1.3 Glycated Haemoglobin............................................................. 14

1.4 Global risk factors for CVD....................................................... 16

1.5 Oxidative stress an overview.................................................... 21

1.5.1 Protein Kinase C and ROS................................................... 22

1.5.2 Oxidative stress in T2DM and CVD................................. 23
1.5.3 Oxidative stress biomarkers, an overview .................23
   1.5.3.1 GSH and GSH/GSSG ..................................24
   1.5.3.2 8 - hydroxy-2′-deoxyguanosine .......................26
   1.5.3.3 F2- Isoprostanes ......................................27
   1.5.3.4 Malondialdehyde ......................................28
   1.5.3.5 Whole Blood Viscosity ................................30
1.6 Inflammation in T2DM and CVD ................................31
   1.6.1 Inflammation – The lipid connection ..................32
   1.6.2 Inflammation and hyperglycaemic states ..............32
   1.6.3 Dyslipidaemia and acute phase response ..............34
   1.6.4 Inflammation and the plaque formation process ......35
   1.6.5 Stress and inflammation ..................................38
1.7 Inflammatory biomarkers ........................................39
   1.7.1 C Reactive Protein ........................................40
   1.7.2 Complement 5a ............................................43
   1.7.3 The interleukins ............................................44
      1.7.3.1 Interleukin - 1β ......................................44
      1.7.3.2 Interleukin – 6 ........................................45
      1.7.3.3 Interleukin - 10 .........................................47
      1.7.3.4 Monocyte Chemoattractant Protein - 1 ............48
1.8 Insulin like growth factor - 1 ..................................50
1.9 Hyperglycaemia and coagulability ............................53
   1.9.1 Thrombus formation .......................................54
      1.9.1.2 Coagulative and fibrinolytic markers ...............55
      1.9.1.3 Homocysteine .........................................55
      1.9.1.4 D-Dimer ................................................57
Conclusion ..................................................................60
Chapter 2 ..................................................................63
   Paper 1: Cardiovascular risk assessment in prediabetes: A
      hypothesis ..........................................................63
Chapter 3 ..................................................................70
Paper 2: Glutathione:Glutathione sulfide redox imbalance in early impaired fasting glucose .............................................70

Chapter 4 ........................................................................................................79

Paper 3: Diabetes, oxidative stress and cardiovascular risk............79

Chapter 5 ..........................................................................................................88

Paper 4: Hyperglycaemia, oxidative stress and inflammatory markers .................................................................................88

Chapter 6 ........................................................................................................109

Paper 5: Antidiabetic, antihypertensive and statin medication use in metabolic syndrome ..................................................109

Chapter 7 ........................................................................................................126

Paper 6: Interaction of homocysteine, glutathione and 8-hydroxy-2’-deoxyguanosine in metabolic syndrome progression. ......126

Chapter 8 ........................................................................................................140

Paper 7: Low serum creatinine levels as risk factor of diabetes mellitus: prediabetes considerations ..........................................140

Chapter 9 ........................................................................................................146

Paper 8: Oxidative stress and inflammation associated with decreased fibrinolysis as an early marker for peripheral vascular disease stratification.........................................................146

Chapter 10 .....................................................................................................155

Paper 9: Association of cytokine activity and the Stroop cognitive function test........................................................................155

Chapter 11 .....................................................................................................176
Paper 10: Acute Phase Inflammatory Response to Single-Bout HIIT and Endurance Training: A Comparative Study ............176

Discussion ..................................................................................................................194

References ....................................................................................................................213

Appendices ..................................................................................................................243

Appendix A: Whole blood viscosity determination in diabetes management: perspectives in practice..........................244

Appendix B: Position paper for health authorities: archived clinical pathology data-treasure to revalue and appropriate ..........251

Appendix C: Serum uric acid and albumin levels and estimated glomerular filtration rate: oxidative stress considerations .257

Appendix D: Serum bilirubin and lipoprotein-a: how are these associated with whole blood viscosity? .........................265

Appendix E: Algorithm for whole blood viscosity: Implication for antiplatelet bleeding risk assessment .........................271


Appendix G: Hyperglycaemia, oxidative stress and inflammatory markers ........................................................................286

Appendix H: Antidiabetic, antihypertensive and statin medication use in metabolic syndrome ...........................................307

Appendix I: Interaction of homocysteine, glutathione and 8-hydroxy-2´-deoxyguanosine in metabolic syndrome progression ........................................................................314
Appendix J: Association of Inflammation and Possible Mild Cognitive Decline. Measured by the Stroop Cognitive Function Test ..........................................................329

Appendix K: Acute-Phase Inflammatory Response to Single-Bout HIIT and Endurance Training: A Comparative Study ......335
Certificate of Authorship

I hereby declare that this submission is my own work and to the best of my knowledge and belief, understand that it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged. I agree that this thesis be accessible for the purpose of study and research in accordance with normal conditions established by the Executive Director, Library Services, Charles Sturt University or nominee, for the care, loan and reproduction of thesis, subject to confidentiality provisions as approved by the University.

Name          Eugene George Butkowski

Signature

Date          23 March 2016
Dedication

This thesis is dedicated to my Father Eugene Butkowskyi (1924 – 2008) for his love, sacrificial support and encouragement provided unconditionally to all of his children and his beloved wife, my mother Jeannie (1931 – 2012). Thank you my Pops you are and were always my inspiration.
Acknowledgments

Firstly to Dr Herbert Jelinek, who as my supervisor and more importantly my mentor throughout the whole of this research project, I am extremely grateful. The completion of this project without his support, encouragement and direction, even throughout the tough and challenging times, would not have been possible. I am truly indebted.

To Dr Hayder Al-Aubaidy for his willingness to undertake my co-supervision and provide valuable and informed input into material published as a result of this thesis.

To Ms. Bev de Jong who not only maintained the continual upgrading of the ever burgeoning database but supplied plenty of moral support.

To Mr. Simon McDonald for his invaluable assistance in providing guidance and assistance in statistical analysis.

To Dr Ezekiel Uba Nwose, for his collaboration, encouragement and example to commitment.

To my employer, up to 2014, South West Pathology Service who supported me, provided a career path for my 40 years of employ and enabled me to conduct routine and research testing during my undergraduate and post graduate studies.

To the many patients who were gracious enough to donate their time and samples to enable this research to be undertaken.

To Charles Sturt University for their support, over the decades, for me as both as an undergraduate and post graduate student. Their commitment to external studies has made University studies a reality to many many thousands of appreciative students.
To all those who provided input and assistance into all of the publications produced for this thesis. Your collegiality and valued input made it all possible.

Finally to my beloved wife Carolyn who has supported me and sacrificed so much to enable me to pursue my studies. Without her support and encouragement I could not have completed this thesis. To my children, and now my grandchildren I hope the baton is passed on in whatever God given good and noble race you have been given.
Ethics Statement

The study was approved by the Charles Sturt University Human Ethics Committee, Diabetes Complications Screening and Research, approval number 2006/042 and complies with the standards set out by the Helsinki agreement for human research.
List of Abbreviations

AGE: Advanced glycation end-products
ABPI: Ankle brachial pressure index
Apo: Apolipoprotein
ATP: Adenosine triphosphate
BMI: Body mass index
C5a: complement factor 5a
CAD: Coronary artery disease
CRP: C-reactive protein
CVD: Cardiovascular disease
DM: Diabetes mellitus
EOS: Erythrocyte oxidant stress
FFA: Free fatty acid
Gpx-1: Glutathione peroxidase
GSH: Reduced glutathione
GSSG: Oxidized glutathione
HbA1c: Glycosylated haemoglobin
HCT: Haematocrit
Hcy: Homocysteine
HDL: High density lipoprotein
HO*: Hydroxyl radical
IFG: Impaired fasting glucose
IGT: Impaired glucose tolerance
IL-1β: Interleukin 1 beta
IL-6: Interleukin 6
IL-10: Interleukin 10
IGF-1: Insulin like growth factor 1
LDL: Low-density lipoprotein
MDA: Malondialdehyde
Metabolic Syndrome: MetS
MCP-1: Monocyte chemoattractant protein 1
NADH: Reduced nicotine adenine dinucleotide
NADPH: Reduced nicotine adenine diphosphonucleotide
NIT: Naming interference test
OGTT: Oral glucose tolerance test
OS: Oxidative stress
O_{2}^{-*}: Superoxide radical
PAI-1: Plasminogen-activator inhibitor type 1
PKC: Protein kinase C
PPP: Pentose phosphate pathway
PVD: Peripheral vascular disease
RIT: Reading interference test
RF: Risk factors
ROS: Reactive oxygen species
SOD: Superoxide dismutase
TC: Total cholesterol
THP-1: Human monocytic cell line derived from acute monocytic leukaemia
TG: Triglyceride
TGF-β: Tissue growth factor - β
TNF-α: Tumour necrosis factor - α
WC: Waist circumference
List of Tables

Table 1 NCEP Adult Treatment Panel III ................................................................. 4
Table 2. Physical properties and lipid compositions of lipoprotein class ......... 11
Table 3. Class of Lipoproteins .............................................................................. 12
Table 4. Traditional biomarkers – target and action ......................................... 19
Table 5. Emerging biomarkers – target and action ............................................. 20
Table 6. ATP III MetS criteria and extended CVD risk ................................. 34
List of Figures

Figure 1. Biomarkers associated with the development of atherosclerosis in T2DM and CVD ................................................................. 5

Figure 2. Pathway(s) activation resulting from hyperglycaemia ..................... 8

Figure 3. Schematic diagram of Chylomicron .............................................. 9

Figure 4 Protein glycation ........................................................................ 15

Figure 5. Inflammation in plaque, rupture and thrombus ............................. 37
Publications arising from doctoral research

Papers listed in the order presented in this thesis


Publications listed in Appendices


Publications – Submitted final versions


ABSTRACT

The recognition by global health authorities that Type 2 diabetes (T2DM) and cardiovascular disease (CVD) is a pandemic health issue has brought about an urgent search for better risk predictors of T2DM and CVD. What has also emerged is that the prediabetic state with elevated glycaemia may go undiagnosed, or is detected incidentally during clinical investigation. A current challenge is to predict the development of T2DM and progression to CVD in these patients. Emerging biomarkers reflecting oxidative stress, inflammatory cytokines, and coagulation and fibrinolytic markers are continuing to provide insight into the pathogenicity of the hyperglycaemic state and its comorbidities.

This thesis proposes an investigation into biomarkers (traditional and emerging) related to hyperglycaemia/prediabetes, T2DM and CVD to observe any correlation, concordance or predictive capacity in the hyperglycaemic state. An initial proposal, presented as a hypothesis, was recognising the availability of traditional screening programs for prediction (e.g. Framingham study) of CVD and diabetes (Paper 1). The hypothesis was to present an alternate model of assessment of diabetic macrovascular complication that will discriminate prediabetes and undiagnosed diabetes. Considering the validity of conventional markers the additional emerging biomarkers: glutathione (GSH), malondialdehyde (MDA), D-dimer and homocysteine (Hcy) were originally proposed. Nwose (2007, PhD dissertation) observed that GSH levels with established CVD compared with those that have either established DM or prediabetes was significantly higher (p <0.0001). D-dimer levels were highest in
DM patients (p <0.003), but increased in pre-DM with CVD (p <0.007). MDA and Hcy were also significant in the prediabetic state (p =0.05 and p <0.01 respectively). As differing parameters may reflect alternative biochemical processes we recommended that analysis of the emerging biomarkers be conducted on a larger cohort, and a longitudinal study subjected to a binomial logistic regression in order to refit modelling for CVD risk also be carried out.

To follow through on emerging biomarkers an investigation into the balance between GSH, glutathione disulphide (GSSG) and 8-hydroxy-2´-deoxyguanosine (8-OHdG) and how this was disturbed in patients with impaired fasting glucose was explored (Paper 2). All emerging biomarkers in this and subsequent papers were analysed by enzyme linked immunosorbent assay (ELISA), general biochemistry was conducted at a nationally accredited pathology laboratory and anthropometric data was conducted at the Diabetes Health (DiabHealth) Initiative at Charles Sturt University (CSU). In this study we observed the GSH:GSSG ratio was significantly lower in the impaired fasting glucose group when compared to controls (p =0.04). A pro-oxidant response to mild-moderate hyperglycaemia with a significant rise in oxidative stress was concluded. A further study involved a cross sectional cohort of patients, enrolled at DiabHealth. The cohort was divided with respect to Framingham CVD risk categories.

Inflammatory and oxidative stress markers, anthropometric and general biochemical markers were reviewed (Paper 3). Significant correlations were again observed with GSH:GSSG between control and type 2 diabetes (T2DM) (p <0.05), and IL-6 was significantly increased in T2DM compared to the control and pre-diabetic groups (p <0.05). This research supported a postulate proposed
that a rebound de novo synthesis of erythrocyte GSH is a response to prolonged hyperglycaemia and that raised Interleukin -6 (IL-6), due to increased reactive oxygen species (ROS), is a result of the up regulation of pro-inflammatory mediators.

In paper 4, oxidative stress and inflammatory markers were further explored in increasing blood glucose concentrations with GSH (p <0.001), GSH:GSSG (p <0.05), 8-OHdG (p <0.05) and Interleukin 1β (IL-1β) (p <0.05) providing significant results when 1st (glucose <4.5mmol/L) and the combined 4th and 5th quintile (glucose >6.1mmol/L) groups were compared. Importantly clients in this study were not discriminated on the basis of medication and results, none-the-less, replicated previous findings for screened patients with no comorbidities. This finding was considered important when conducting community based screening as biomarkers need to be informative for all persons being tested and in identifying T2DM and CVD disease progression. Ratios of inflammatory markers were also explored in this study, as a review of the literature revealed no previous investigations had been conducted for their association with hyperglycaemia. IL-1β/IL-10, IL-6/IL-1β and CRP/IL-6 provided significant results (p <0.05), affirming ratios could be included in routine investigations.

As paper 4 did not exclude patients on the basis of medication use in the outpatient community, a study on medication use with respect to metabolic syndrome (MetS) (as defined by ATPIII) was investigated (Paper 5). Antidiabetic, antihypertensive and Statin use differed significantly between a MetS and No MetS group defined by ATPIII (p <0.0001). When medication use was separated into anti-diabetic, anti-hypertensive and statins, similar significant differences were found between the Metabolic Syndrome (MetS) and No MetS
The focused outpatient community showed medication is relatively well controlled for MetS, however statin use in the MetS group indicated usage may be below that recommended.

The investigation in paper 6 further tested the role of homocysteine, which remains a controversial biomarker, for disease progression and associated oxidative stress processes in the categories of no MetS (<3 factors) vs MetS (≥3 factors). A caveat of this study was to include antihypertensive, antihyperlipidaemic and antihyperglycaemic medication, which are not part of the ATPIII definition for MetS. Hcy (p =0.03) and 8-OHdG (p =0.0001) were significantly elevated in the MetS group compared to the no MetS. The conclusion was the significant Hcy result and clustering of Mets factors are likely the result of complex metabolic pathophysiology interactions associated with 8-OHdG and Hcy.

Traditional markers have an important place in aiding diagnosis. Increased or decreased creatinine, a traditional renal function marker, has been postulated as a risk factor for T2DM. In paper 7, 102 patients derived from a pathology data base archive was categorised into three groups premised upon glucose tolerance test results i.e. Control (No diabetes), Prediabetes and Diabetes. Glomerular filtration rate (GFR) and serum creatinine results were analysed using ANOVA and post-hoc analysis with no significant difference observed at any level. We concluded that low creatinine should not be used as a T2DM predictive biomarker pending further investigation and clarification on a larger cohort.

Peripheral vascular disease (PVD), possibly a first indicator of atherosclerosis was investigated in paper 8. Arterial stenosis and calcified arteries can be generally differentiated according to low or increased ankle brachial pressure
index (ABPI) respectively. Oxidative stress and inflammatory markers were measured to observe any differences between low (<1.07) and high (>1.23) ABPI determined as the cut-off for the 1st and 3rd tertile. D-dimer (p <0.001) and GSH (p <0.05) were significant between groups. C-reactive protein (CRP), D-dimer and IL-6 combined with traditional biomarkers BGL, HbA1c and systolic blood pressure were the best model for predicting ABPI class (p<0.05). The results suggest that emerging oxidative stress and inflammatory biomarkers are important contributors in identifying different pathophysiological processes involved in peripheral vascular pathophysiology.

Cognitive decline as a consequence of T2DM is recognised, however mild cognitive and inflammation is not conclusive. In paper 9 participants undertook the Stroop testing battery i.e. reading and naming interference tests (RIT and NIT) and inflammatory markers were measured on each patient. CRP was significant for RIT (p = 0.022); IL-1β (p = 0.039), MCP-1 (p <0.01) was significant for NIT. IL-10 (p <0.01); IL-6/IL-10 (p <0.03) were significant in RIT and NIT. This is the first study to demonstrate a connection between inflammatory markers and behavioural data.

Exercise is known to reduce comorbidities in T2DM. In paper 10 the effect of endurance testing (ET) and high-intensity interval training (HIIT) on inflammatory cytokines and IGF-1 were tested on young adults. Blood was drawn before the interventions, 30 min and 2 days after the training sessions. Significant results were obtained with IL-6/IL-10 ratio (p =0.047), and a decrease of MCP-1 (p =0.03). HIIT may present a valid alternative to ET which has implications on obesity derived from inactivity on populations where ET is not an option.
The research conducted in this thesis has contributed to the expansion of our understanding of the role oxidative stress and inflammatory biomarkers have in the hyperglycaemic state, T2DM and CVD. Further evaluation of emerging biomarkers by increasing participant numbers and providing a longitudinal study is recommended in our efforts to obtain more informative and predictive information in the hyperglycaemic state.
Chapter 1
Emerging biomarkers of oxidative stress and inflammation as indicators of T2DM progression and association with CVD risk

1. General overview of the issue:

Oxidative stress and inflammatory processes are now considered to be a common thread in many disease processes inclusive of obesity, diabetes, atherosclerosis and cardiovascular disease. Conventional risk prediction algorithms may be available but authentic and accurate biomarkers remain lacking (Upadhyay, 2015). The purpose of this thesis was to investigate emerging biomarkers indicative of oxidative stress and inflammation markers in hyperglycaemic and cardiovascular disease states.

Whilst it is viewed that cardiovascular disease (CVD) complications of diabetes is a major worldwide health problem, knowledge of the progression of Type 2 diabetes (T2DM) and degree of CVD risk associated with T2DM requires further exploration. So profound and critical to global health is the impact of CVD, including heart disease, vascular disease and atherosclerosis, that it is purported to contribute to over one third of the global morbidity (Das & Chakrabarti, 2006). On this basis alone national, global and private funding bodies to health care should continually review their commitment to all necessary support into the investigation and research of this insidious pathology. CVD a major complication which may result from T2DM is the leading cause of early disability and death among diabetics. T2DM is now viewed as a pandemic clinical disorder with no distinction between developing and developed countries, and continues to contribute to the burden of CVD (Neergheen-
Bhujun, 2014). In the United States it has been estimated that by 2006 CVD was of epidemic proportions afflicting some 81.1 million and claiming the lives of 831,000 (Bhamidi, 2011). Other affluent countries, Australia included, now appear to be mirroring this pre-diabetic, T2DM and CVD state (AIHW, 2014). A major concern is that as many countries emerge from third world conditions with an ensuing increase in a middle class population there will be a further exacerbation in this insidious disease state. In 2006 it was reported in the Daily Sun that some 12 million Nigerians were now suffering from diabetes (Agwuna, 2006). This would indicate that we are not necessarily dealing only with a lifestyle epidemic but other factors are also contributing. The early genesis of this thesis, in recognition of the morbidity attributed to T2DM and CVD, lead to our medical hypothesis on a cardiovascular risk assessment in prediabetes (Nwose, Richards, Cann, & Butkowski, 2009).

The heterogeneous nature of T2DM contributes to the diverse views of how elevated levels of glucose contribute to the endothelial dysfunction evident in CVD associated with T2DM. Strong epidemiology evidence exists that T2DM is a major risk factor for CVD with several plausible mechanisms underlying their association and underpinning possible treatment regimen (Desouza, Raghavan, & Fonseca, 2010). While the diagnostic capability of elevated blood glucose per se is a predictor of DM (types 1 & 2) and to some extent insulin resistance, it is relatively a poor predictor of CVD. Cardiac autonomic dysfunction, assessed by heart rate variability (HRV), is a known risk factor for the development of CVD (Hillebrand et al., 2013) and association with hyperglycaemia and a decrease in HRV has been demonstrated (Tarvainen, Lipponen, Al-Aubaidy, & Jelinek,
There is however a significant relationship between blood glucose and CVD but no reliable threshold level of glucose and the identification with CVD (Kelly et al., 2009). Some microvascular complications, in particular retinopathy, do evidence a clearer threshold with glycaemia (d’Emden et al., 2012). The effects of hyperglycaemia are multifactorial, as HRV is subject to autonomic control and structurally the cardiovascular system is impacted by the OS and inflammatory response. These effects further reinforce the purpose of how OS and the inflammatory mechanisms participate in hyperglycaemia and CVD and the importance of conducting the analysis of these emerging biomarkers. In addition prediabetes and metabolic syndrome could not be excluded from this thesis as they form an integral part of the hyperglycaemic continuum.

The World Health Organisation (WHO) has long recognised the link between obesity, CVD and T2DM and has classified a constellation of factors to include at least three of the five characteristics (Table 1) to describe ‘Metabolic Syndrome’ (MetS) (S. Haffner & Taegtmeyer, 2003; Kereiakes & Willerson, 2003; Reilly & Rader, 2003). The MetS risk for the development of CVD and T2DM is dramatically increased in people which meet the diagnostic criteria for MetS (P. W. F. Wilson, D’Agostino, Parise, Sullivan, & Meigs, 2005) – Table 1 emphasises the National Cholesterol Education Program (NCEP) Adult Treatment Panel III:
<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Defining Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal obesity</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>&gt;102 cm</td>
</tr>
<tr>
<td>Women</td>
<td>&gt; 88</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>&gt;1.7 mmol/L</td>
</tr>
<tr>
<td>HDLC</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>&lt;1.0 mmol/L</td>
</tr>
<tr>
<td>Women</td>
<td>&lt;1.3 mmol/L</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>&gt;130/80 mm Hg</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>&gt;6.1 mmol/L</td>
</tr>
</tbody>
</table>

> 3 risk factors is indicative of MetS

Figure 1 was developed to portray current understanding of the biochemistry and pathophysiology including the oxidative stress, inflammation and coagulation processes involved in the development of T2DM, CVD and atherosclerosis. It builds upon the molecular events that occur due to the presence of hyperglycaemia.
This figure depicts the formation of the atherosclerotic lesion linking the biochemical and pathophysiological changes which occur due to the interactions of oxidative stress, inflammation and coagulation/fibrinolysis. The pathology is linked directly to T2DM and CVD.

1.1 Atherosclerosis

T2DM is considered to be a major risk factor for the development of atherosclerosis, often pre-eminent in both T2DM and CVD. Atherosclerotic
lesions are now considered to be tissue assault as a result of oxidative stress, endothelial dysfunction and smooth muscle cell migration and proliferation (Mehta, Rasouli, Sinha, & Molavi, 2006).

Until more recently the standard markers in assessing atherosclerosis were the use of low density lipoprotein (LDL), high density lipoproteins (HDL) and triglyceride levels (TG), however it is recognised that many future vascular events may occur without any evidence of overt hyperlipidaemia (Ridker, Brown, Vaughan, Harrison, & Mehta, 2004). An early recognition of the evidence of atherosclerosis is the presence of foam cells (lipid laden macrophages), originating from circulating monocytes and poorly understood precipitating molecular mechanisms (Duthie, Wahle, & James, 1989; Schrijvers, De Meyer, Herman, & Martinet, 2007).

It is now well accepted that atherosclerosis is not merely a lipid disorder, but also a chronic inflammatory disease (Russell Ross, 1999). Inflammation is recognised as a major contributor to atherogenesis through adverse effects on lipoprotein metabolism and it has also been long established that improving glycaemic control is associated with preventing the progression of microvascular complications in T2DM (Mannucci, Dicembrini, Lauria, & Pozzilli, 2013).

Brownlee has stated in his pioneering and pivotal work of oxidative stress as a unifying factor in the etiology of diabetic complications that there are four associated mechanisms, namely: hyperglycaemia increases flux through the polyol pathway; the intracellular production of advanced glycation end products (AGE); the activation of protein kinase C (PKC) and an increase in hexosamine activity (Brownlee, 2005; Sheetz & King, 2002). Figure 2 portrays the four key biochemical pathways activated by hyperglycaemia induced oxidative stress. The
increased flux of glucose through the polyol pathway has been implicated in DM. During hyperglycaemia, the saturation of hexokinase by glucose renders the availability of glucose to the rate limiting polyol pathway enzyme aldose reductase. Via this pathway glucose is reduced to sorbitol with further dehydrogenation by sorbitol dehydrogenase to fructose. The slowly metabolised sorbitol is not readily diffusible through cell membranes and accumulates within the cell, the intracellular increase in osmotic stress leads to an influx of water (Berrone, Beltramo, Solimine, Ape, & Porta, 2006). Fructose provides a similar result. Decreased levels of NADPH as a result of the activated polyol pathway also depletes nitric oxide (NO) and glutathione (GSH) resulting in further ROS. For more discussion on GSH refer to the oxidative stress GSH section, however the interaction between hyperglycaemia, oxidative stress and inflammation is central to this thesis and therefore cross referencing will occur due to the lack of exclusivity. The link between lower levels of GSH, is brought about by two different mechanisms. The decrease can occur through the direct effect of glucose and insulin on GSH synthesis and also on the consumption of the cofactor NADPH via the polyol (Ballatori et al., 2009) (Fig 2). Further comment is made in the oxidative stress section and is pursued in our publications on oxidative stress in T2DM, CVD, MetS and comorbidity studies.
Figure 2: Pathway(s) activation resulting from hyperglycaemia

This process results in reduced levels of NADPH, GSH, increased AGE formation, activation of PKC (expression of proinflammatory genes, vascular occlusion) and flux of excess glucose through the hexosamine pathway (increased transcription of inflammatory cytokine genes) (A. Ceriello, 2011).

1.2 Lipid metabolism and contribution to T2DM and CVD

Dyslipidaemia is a common entity observed in both T2DM and CVD. The biochemical interactions and pathophysiology of T2DM and CVD is both complex and multifactorial with oxidative interactions contributing to the development of atherosclerosis (A. Ceriello, 2011; Krauss, 2004). Following is a review of lipid biochemistry and lipid classification.

1.2.1 Lipid biochemistry

Elevated plasma lipid levels, particularly cholesterol are causally related to the pathogenesis of atherosclerosis, the process responsible for the majority of CVD. Pioneering works such as the Framingham study have demonstrated the
epidemiology and associated causal links (Dawber & Kannel, 1966). The major lipids present in the plasma are fatty acids, triglycerides, cholesterol and phospholipids. To a lesser degree but of major physiological importance are the steroid hormones and fat soluble vitamins (Marshall, 2004).

**FIGURE 3. SCHEMATIC DIAGRAM OF CHYLMICRON**

Nascent chylomicrons are secreted by the intestinal cells and are transported via the lymphatic system to the blood, where they will pick Apoprotein C-II and Apo E. Apolipoprotein C-II, as part of chylomicron, activates Lipoprotein Lipase, an enzyme attached to the luminal surface of the endothelial cells of capillaries. This enzyme catalyzes the hydrolysis of the triacylglycerols (triglycerides) in the chylomicron. ApoB 48, a component of Apo B, a unique protein to chylomicrons from the small intestine, returns to the liver as part of the chylomicron remnant, where it is endocytosed and degraded.

### 1.2.2 Lipid classification

Altered lipid profiles in T2DM are likely to contribute to the atherosclerotic process, with HDL levels often decreased and LDL increased. The increased
catabolism of HDL, often a consequence of hypertriglyceridaemia, and subsequent modified LDL metabolism presents the oxidative stress, inflammatory cascade (Verges, 2009). An understanding of lipid metabolism is crucial to our understanding of T2DM, CVD and atherosclerosis.

Lipoprotein metabolism is complex with abnormal concentrations of various lipoprotein particles being formed from rates of production, conversion or catabolism of lipids and proteins. The implications of altered states in lipid metabolism (diabetic dyslipidaemia) and their associated risk of CVD may account for some of the complex nature of altered states associated with artherogenic lipid and lipoprotein abnormalities (Borén & Adiels, 2014).

The non-soluble cholesterol and triglyceride molecules are transported within the plasma as part of water soluble lipoprotein macromolecules. The cholesterol is present in two forms: free cholesterol, a polar non esterified alcohol and cholesterol ester, a hydrophobic form wherein the cholesterol is bound to free fatty acid. The lipoprotein structural arrangement (micelle) places the hydrophobic lipids at the core of the micelle whereas the water soluble free cholesterol and phospholipids are arranged on the surface with the polar groups orientated outwards (Fig 3) (Hilbert, 2007).

Traditionally lipoproteins have been differentiated according to their ultracentrifugation properties. Ideally, the lipoprotein aggregates should be described in terms of the different protein components or apolipoproteins, as these determine the overall structures and metabolism, and the interactions with receptor molecules in liver and peripheral tissues. However, the practical methods that have been used to segregate different lipoprotein classes have determined the nomenclature. Thus, the main groups are classified as
chylomicrons (CM), very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL), based on the relative densities of the aggregates on ultracentrifugation. However, these classes can be further refined by improved separation procedures, and intermediate-density lipoproteins (IDL) and subdivisions of the HDL (e.g. HDL1, HDL2, HDL3 and so forth) are often defined. Each of these subdivisions may have distinctive apolipoprotein compositions and biological properties that may be pertinent to CVD. LDL itself is not a homogenous category of lipoproteins but a set of discrete subclasses dependent upon their molecular properties. In normal population studies these LDL subfractions are categorised as I, IIa, IIb, IIIa, IIIb, IVa, IV (Superko, 2009). Density of the lipoproteins is determined largely by the relative concentrations of triacylglycerols and proteins and by the diameters of the broadly spherical particles, which vary from about 6000Å in CM to 100Å or less in the smallest HDL. An alternative nomenclature is based on the relative mobility’s on electrophoresis on agarose gels. Thus, α, pre-β and β lipoproteins correspond to HDL, VLDL and LDL, respectively (AOC, 2014b); compositional details are listed in Table 2.

**Table 2. Physical properties and lipid compositions of lipoprotein class**

<table>
<thead>
<tr>
<th></th>
<th>CM</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (g/ml)</td>
<td>&lt; 0.94</td>
<td>0.94-1.006</td>
<td>1.006-1.063</td>
<td>1.063-1.210</td>
</tr>
<tr>
<td>Diameter (Å)</td>
<td>6000-2000</td>
<td>600</td>
<td>250</td>
<td>70-120</td>
</tr>
<tr>
<td>Total lipid (wt %) *</td>
<td>99</td>
<td>91</td>
<td>80</td>
<td>44</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>85</td>
<td>55</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Cholesterol esters</td>
<td>3</td>
<td>18</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>2</td>
<td>7</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>8</td>
<td>20</td>
<td>29</td>
<td>46</td>
</tr>
</tbody>
</table>

CM – chylomicrons, VLDL – very low density lipoprotein, LDL – low density lipoprotein, HDL – high density lipoprotein

* Most of the remaining material comprises the various apolipoproteins.

(Adapted from: [http://lipidlibrary.aocs.org/Lipids/lipoprot/index.htm](http://lipidlibrary.aocs.org/Lipids/lipoprot/index.htm))
1.2.2.1 Apolipoproteins

The Apolipoproteins constitute the major protein component of lipoproteins and are commonly referred to by a standard nomenclature introduced by (Alaupovic, 1971). The lipoproteins have a variety of functions, which include structural components of lipoprotein particles in lipid transport and cofactors for enzymes and ligands for cell surface receptors (Saito, Lund-Katz, & Phillips, 2004). Table 3 depicts the class of apolipoproteins and their association with the major functional lipoproteins (Rader, Hoeg, & Brewer, 1994).

<table>
<thead>
<tr>
<th>Apolipoproteins</th>
<th>Major Lipoproteins</th>
<th>Major Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo A-1</td>
<td>HDL</td>
<td>Structural protein for HDL, activator of LCAT</td>
</tr>
<tr>
<td>Apo A-II</td>
<td>HDL</td>
<td>Structural protein for HDL, activator of hepatic lipase</td>
</tr>
<tr>
<td>Apo A-IV</td>
<td>HDL, chylomicrons</td>
<td>Activator of LPL and LCAT</td>
</tr>
<tr>
<td>Apo B-100</td>
<td>VLDL, IDL, LDL</td>
<td>Structural protein for VLDL and LDL, ligand for binding to LDL receptors</td>
</tr>
<tr>
<td>Apo B-48</td>
<td>Chylomicrons, remnants</td>
<td>Structural protein for chylomicrons</td>
</tr>
<tr>
<td>Apo C-II</td>
<td>Chylomicrons, VLDL</td>
<td>Essential cofactor for LPL</td>
</tr>
<tr>
<td>Apo C-III</td>
<td>Chylomicrons, VLDL, HDL</td>
<td>Inhibitor of lipoprotein binding to receptors</td>
</tr>
<tr>
<td>Apo E</td>
<td>Remnants, LDL, HDL</td>
<td>Ligand for binding to receptor, ligand for binding to remnant (Apo E) receptor</td>
</tr>
<tr>
<td>Apo (a)</td>
<td>Lp(a)</td>
<td>Structural protein Lp(a), inhibitor of plasminogen activation</td>
</tr>
</tbody>
</table>

Apo - apolipoprotein, HDL - High density lipoprotein, IDL - intermediate lipoprotein, LCAT - lecithin-cholesterol acyltransferase, LDL - low density lipoprotein, Lp(a) - lipoprotein(a), LPL - lipoprotein lipase; VLDL - very low density lipoprotein

Many studies have reviewed the association of certain apolipoprotein isoforms with CVD risk and the contribution to the inflammatory process. For example
the smaller Apo A molecules appear to have an approximate 2 fold increase in risk for coronary heart disease (Erqou et al., 2010). Although circulating lipoprotein(a) (Lp[a]) is likely to be a causal risk factor in coronary heart disease (CHD), the magnitude of this association is modest. Lp(a) particles with smaller, rather than larger, apo(a) isoforms may be stronger risk factors however previous studies concerning apolipoprotein A-I (apo A-I), apolipoprotein B (apo B), and Lp(a) in retrospective cross-sectional studies, have shown apo A-I levels were not substantially more predictive of CHD than were high-density lipoprotein (HDL) cholesterol levels. In contrast, levels of apo B and Lp(a) were often more strongly associated with coronary artery disease than were traditional lipid measurements (Rader et al., 1994). Lp(a), an indicator of oxidative stress, has emerged as a potential risk factor for CVD and has been shown to attenuate fibrinolysis. However an investigation into any link with whole blood cell viscosity (WBV), a known sensitive marker of oxidative stress, was not proven warranting further research (Nwose, Richards, Bwititi, & Butkowski, 2012). Apo E (2 and 4 isoforms) are associated with Type 3 hyperlipoproteinaemia and CHD respectively (Hilbert, 2007). Much of the esoteric nature of this lipid discussion however does underlie the importance that the traditional markers TC, HDL, LDL and TG, tested and statistically evaluated in my studies, support previous findings indicating the impact of lipid studies and their association with T2DM and CVD post Framingham cannot be underestimated. What is problematic is their use as a predictor in the pre-diabetic/hyperglycaemic state is still very much subjective.
1.3 Glycated Haemoglobin

Glycated haemoglobin (HbA1c) measurement as an alternative and adjunct test for glucose monitoring is now well established as a proven method in assessing and monitoring diabetic patients. Its utility relates to its ability to represent average long term glucose concentration. Normal levels of glucose in effect produce a normal level of HbA1c whereas elevated levels increase the glycosylation of Haemoglobin in a predictable way. The normal life span of a red blood cell is approximately 100 – 120 days; therefore raised blood glucose will increase the level of HbA1c proportionally over this time period and will be reflected accordingly.

In vivo, glucose normally predominates as a cyclic molecule which is in equilibrium with a small fraction of the acyclic form. The glycation of proteins (including haemoglobin) commences with the acyclic forms chemically active aldehyde group which can react non-enzymatically with amino groups. This two-step reaction occurs fairly rapidly first and forms a reversible aldimine - Schiff base. This is followed by a slower formation of the aldimine to the irreversible ketoamine (Fig 4). The glycation process therefore exists for the life of the protein molecule, making it extremely useful in monitoring longer term hyperglycaemia. Interestingly in DM patient’s LDL particles undergo a similar glycation process to HbA1c which lengthens the half-life of LDL and increase its ability to promote atherogenesis. Paradoxically the glycation of HDL shortens its half-life thus diminishing its protective ability (Dokken, 2008).
It is widely accepted that the efficacy of HbA1c as a suitable marker for monitoring glycaemic control as a diagnostic tool for DM is due to its high level of analytical sensitivity and specificity. It is therefore considered to be a prominent bio marker for the long term prediction of complications in DM (DCCT., 1993).

What is still problematic however, along with the recognition that CVD is the greatest cause of death in DM the risk of suffering a CVD event is increased two to fourfold in DM patients (Laakso, 2001). To aid in the prevention of microvascular and macrovascular it is recommended that HbA1c concentrations should remain less than 7.0% (Hinzmann, Schlaeger, & Tran, 2012). HbA1c therefore still posit its main utility in the prediction, diagnosis and management in DM rather than as a stand-alone biomarker for microvascular and/or macrovascular changes. A recent study conducted by ‘The Emerging Risk Factors, Collaboration’ has determined that HbA1c monitoring does not provide any clinically meaningful risk assessment and supports the conclusion of the American College of Cardiology/American heart Association Guideline on the
Assessment of Cardiovascular Risk (The Emerging Risk Factors, 2014). There may possibly be an algorithmic association with some traditional biomarker predictors of CVD potentially providing some useful information (Simmons, Sharp, Boekholdt, & et al., 2008; U.S. Preventive Services Task Force, 2002), however adding FBG or HbA1c may not improve predictions in non-diabetes (Schottker, Muller, Rothenbacher, & Brenner, 2013). Inflammatory markers and OS may therefore improve predictability and are pursued and discussed further in subsequent publication chapters. Substantial hypoglycemia should also be avoided due to an association of low blood glucose and CVD events (Moghissi et al., 2009) and should be borne in mind when evaluating statistical evaluation of emerging biomarkers for CVD prediction, particularly in light of the wide acceptance and usage of HBA1c as a screening test for DM. When considering the individual components of oxidative stress, inflammation and hypercoagulability states it is important to recognise through this discussion that they cannot be considered as mutually exclusive, albeit categorised separately. When considering hyperglycaemia, transduction pathways warrant a brief discussion to aid our understanding of oxidative stress.

1.4 Global risk factors for CVD

The Framingham Risk Assessment and the New Zealand Risk Assessment Protocols currently have a 20% false negative rate (Murabito et al., 2003) requiring further research to identify possible novel and emerging biomarkers to reduce the false negative rate. The role biomarkers play as predictors of T2DM and CVD is as diverse as it is controversial. A number of CVD risk models have
been developed over the years in addition to the Framingham study, including the Systematic Coronary Risk Evaluation (SCORE), and Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria in Europe (DECODE), where T2DM groups have been represented as subgroups of population studies but have not included HbA1c and DM duration as continuous risk variables with a demonstrable inability in providing reliable fatal CVD and CHD risk estimates in T2DM (Coleman, Stevens, Retnakaran, & Holman, 2007).

Other studies such as the Swedish National Diabetes Register have examined statistical predictors which have followed a longitudinal study based on history of DM, systolic BP, HbA1c, BMI, sex, anti-hypertensive therapy, lipid lowering drugs and tobacco use to monitor the development of CVD in DM patients. This five year predictive equation incorporated non laboratory predictors and HbA1c providing good discrimination between observed and predicted risk (Cederholm et al., 2008). Abbasi et al. identified a number of existing prediction models for T2DM, recognising that of 25 prediction models from a Dutch cohort it showed that the basic models faired similarly in identifying high and low risk individuals at developing T2DM. Slightly more optimal results were obtained when conventional biomarkers such as glucose, HbA1c, lipids, uric acid or γ-glutamyl transferase were included, with all but two overestimating the risk of DM development (Abbasi et al., 2012). The emerging biomarkers studied in this thesis, as presented in the publications, were chosen to provide more cogent predictability determining the risk, or progression to T2DM.

Without disputing the efficacy of any of these findings the Holy Grail being sought is in the identification of a reliable biomarker(s) in identifying a predictor of T2DM and progression to CVD to assist in their prevention and timely
A more recent extensive review by Herder et al. on risk prediction for T2DM and CVD has identified the current suboptimal information obtained upon review of biomarkers from genomics, transcriptomics, proteomics, and metabolomics, as well as serial measurements of biomarkers. (Herder, Karakas, & Koenig, 2011). The lack of conclusive evidence in genotypes and some biomarkers as sole predictors would require a complete assessment of all parameters included in Herders review. Witzel et al. have conducted an extensive study of genetic variants showing significant association with diabetic neuropathies, for example ACE, GPx1, IL-4, IL-10 to name just a few. (Witzel et al., 2015),

It is evident that increased knowledge of the emerging biomarkers in this study requires further investigation if a global predictive algorithm is obtainable. A recent study by the author investigated inflammatory and oxidative stress markers in addition to the factors associated with the Framingham risk equation and observed significant results for a non-traditional (novel and emerging) biomarker IL-6 between control and T2DM and Pre-DM and T2DM and the oxidative stress marker GSH in moderate and high risk CVD risk groups (Butkowski, Brix, Al-Aubaidy, Kiat, & Jelinek, 2016). The importance in these findings was the demonstration that some single biomarkers are indicative of disease progression and that there are possible interactions between inflammation and oxidative stress. An outcome for follow up from the data generated by this thesis will be an examination of the existing data plus the inclusion of more biomarker investigations to provide greater patient numbers to enable the possible generation of a predictive algorithm for T2DM and/or CVD progression. It would be anticipated that the modelling for the algorithm would be based upon
OS and inflammatory biomarkers. The following sections provide a discussion of OS, inflammation and coagulation and their respective markers. Table 4 provides a list of the traditional markers investigated and Table 5 lists the emerging biomarkers.

### Table 4. Traditional Biomarkers – Target and Action

<table>
<thead>
<tr>
<th>APR</th>
<th>Cell source</th>
<th>Target</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>APR</td>
<td>APR</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CRP</th>
<th>Liver</th>
<th>Tcell, macrophages</th>
<th>Inflammatory response</th>
</tr>
</thead>
</table>

**Cell injury, immunity**

<table>
<thead>
<tr>
<th>C5a</th>
<th>Liver, macrophages</th>
<th>Endothelium, smooth muscle, phagocytes, mast cells</th>
<th>Anaphylotoxin, innate immunity, chemoattractant, coagulation</th>
</tr>
</thead>
</table>

**Oxidative stress**

<table>
<thead>
<tr>
<th>MDA</th>
<th>Product of lipid peroxidation</th>
<th>All cells</th>
<th>Oxidative stress injury</th>
</tr>
</thead>
</table>

APR – acute phase reactant, C5a – complement 5a, MDA - malondialdehyde

Table 4 lists the main traditional markers of interest in this study.
Table 5. Emerging Biomarkers – Target and Action

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Target</th>
<th>Actions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro-inflammatary Cytokine markers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1(β)</td>
<td>Macrophage, dendritic cell</td>
<td>Lymphocytes, endothelial cells, CNS, liver</td>
</tr>
<tr>
<td>IL-6</td>
<td>Macrophage, dendritic cells, endothelium, Th 1 cells</td>
<td>Liver, B cells</td>
</tr>
<tr>
<td>MCP-1 (chemotactic cytokine)</td>
<td>Monocytes, macrophages (major sources)</td>
<td>White Blood cells and haemopoietic cells</td>
</tr>
<tr>
<td><strong>Anti-inflammatary Cytokine marker</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>Macrophage, Th2 cells</td>
<td>Macrophage, dendritic cells</td>
</tr>
<tr>
<td><strong>Hormone marker</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-1</td>
<td>Hepatocytes</td>
<td>All cells</td>
</tr>
<tr>
<td><strong>Oxidative stress markers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>All cells, primarily hepatic</td>
<td>Substrate for conjugation and reduction reactions</td>
</tr>
<tr>
<td>GSSG</td>
<td>All cells, primarily liver (oxidised GSH)</td>
<td>Oxidised GSH</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>Product of DNA – deoxyguanosine oxidative damage</td>
<td>All cells</td>
</tr>
<tr>
<td>F2 -Isoprostane</td>
<td>Free radical catalysed peroxidation of fatty acids (arachidonic)</td>
<td>All cells</td>
</tr>
</tbody>
</table>

IL-1β – Interleukin 1 beta, IL-6 – Interleukin 6, MCP-1 – Monocyte chemotactic protein 1, IL-10 – Interleukin 10, IGF-1 – Insulin like growth factor 1, GSH – Glutathione, GSSG – Glutathione disulphide, 8-OHdG – 8 hydroxy-deoxy-guanosine,

Table 5 lists the emerging biomarkers of inflammation and oxidative stress studied.
1.5 Oxidative stress an overview

Oxidative stress (OS) may be described as a disturbance in the ‘oxidant/antioxidant’ balance within the cell, where the oxidant prevails (Sies, 1991). It is a state in which a cell is experiencing alteration of cellular components, due to its being under high exposure to free radicals and reactive oxygen species (ROS) beyond its antioxidant capacity. Though, the reaction of ROS are essential for cellular functions such as the utilisation of the chemical energy of nutrients for the production of adenosine triphosphate (ATP), excess oxidant exposes the cells involved to OS. The generation of oxygen free radicals is often formed through the stimuli of physical agents such as chemical, radiation, pollutants and physiological processes which have the capacity to disrupt mitochondrial function (Lobo, Patil, Phatak, & Chandra, 2010; K. Rahman, 2007).

Whilst the literature portrays ample information in regard to the presence of oxidative stress in a variety of pathophysiological processes, the mechanisms of oxidative stress is still under constant review and more research is required for further elucidation and understanding. The clear correlation between many disease states and oxidative stress is still lacking due to insufficiencies in the full understanding of contributing molecular mechanisms. An example we pursued (see appendix c) was to observe if a relationship existed with T2DM, kidney function and the oxidant/antioxidant - uric acid and albumin which is known to have antioxidant capabilities (Bwititi et al., 2012). Uric acid and eGFR were significant (p<0.001), along with a weak partial correlation once corrected for age, our findings indicated that extensive studies are still required, considering
that raised uric acid levels may be a result of renal impairment and not directly related to oxidative stress as a consequence of hyperglycaemia. It is probable that uric acid may contribute more as an oxidant. As renal function is a major clinical morbidity in T2DM and CVD oxidative stress markers and renal function may be important when considering the impact of biomarkers reviewed in this thesis. This is further supported by studies with urinary microalbuminuria as correlations with increasing levels are a known indicator of progressive renal nephropathy in diabetes and significant increases have also been observed in prediabetic groups (Alsaad & Herzenberg, 2007). In a recent study an improvement in eGFR was noted in T2DM and CVD patients possibly due to treatment with statins (Butkowski et al., 2016; Foundation, 2006). GSH, GSH/GSSG, 8-OHdG, MDA, F₂ – isoprostane and whole blood viscosity are discussed in this section (see 1.4.3.1)

1.5.1 Protein Kinase C and ROS

All forms of diabetes are characterised by hyperglycaemia. As a consequence the diabetic metabolic abnormalities result in endothelial superoxide production, which in turn produces pathological processes including the activation of the Protein Kinase C isoforms (Giacco & Brownlee, 2010) (see Fig 2). The family groups of Protein Kinase C (PKC) which feature in many cellular signal transduction pathways, are enzymes involved in the control of protein functionality through the phosphorylation of the hydroxyl groups of the residue groups of threonine and serine. ROS may trigger various PKC isoforms (PKC-α, β1/2 and PKC – δ), implicated in CVD, through redox signaling and has been
associated with the vascular alterations related to permeability, contractility, extracellular matrix synthesis, cell growth and apoptosis, angiogenesis, leukocyte adhesion and cytokine activation and inhibition (De Marchi, Baldassari, Bononi, Wieckowski, & Pinton, 2013; C. Huang & Freter, 2015).

1.5.2 Oxidative stress in T2DM and CVD

Oxidative stress is likely implicated in diabetes and vascular disease states. The endothelium plays an integral role in many disease states by regulation of vascular tone, platelet activity, leucocyte adhesion and thrombosis (Rajendran et al., 2013) and can therefore be considered to be intricately linked to the development of atherosclerosis. Increasing evidence is highly suggestive of oxidative stress contributing significantly to endothelial dysfunction (Nwose, Richards, & Bwititi, 2014). Increased production of oxygen-derived free radicals such as the superoxide anion has been linked to impaired endothelial vasomotor function in clinical and experimental models of atherosclerosis. (Heitzer, Schlinzig, Krohn, Meinertz, & Munzel, 2001).

1.5.3 Oxidative stress biomarkers, an overview

It is widely accepted that oxidative stress plays a central role in the pathophysiology of T2DM and alterations to the vascular and neurological changes, possibly via mediating the diversion of glycolytic intermediates (McGrowder, Anderson-Jackson, & Crawford, 2013). The following discussion provides a review of traditional and novel oxidative stress markers. The
association of whole blood viscosity (WBV) with diabetes and oxidative stress also warrants a brief discussion (further publications in this area are included in the appendices).

1.5.3.1 GSH and GSH/GSSG

Reduced glutathione, one of the major cellular antioxidants, is a linear tripeptide consisting of L-glutamine, L-cysteine and glycine possessing a sulphydryl (SH) group on the cysteinyl portion accounting for its strong proton donating character. The two step synthesis of the tripeptide is catalysed by L-glutamyl-L-cysteine: glycine ligase and glutathione synthetase (Sekhar et al., 2011). The biological activity of GSH resides in the proton donor sulphydryl group of the cysteine moiety; the loss of the GSH electrons result in the oxidised form, consisting of a reversible linked dimerised disulphide (GSSG). A tight homeostatic control exists, both intracellular and extracellular, with a dynamic balance maintained between GSH synthesis (Godoy et al., 2013), the recycled GSSG/oxidised GSH and its utilisation (Owen & Butterfield, 2010).

Despite the diverse nature of systemic issues with T2DM and the recognition of the multifactorial pathways involved in mediating tissue damage a common feature is increased oxidative stress marked by demonstratively elevated levels of ROS. The generation of oxidative stress and increased ROS in hyperglycaemia appears to be diminished by lowering the concentration of blood glucose. Notably intracellular cysteine and glycine have also been demonstrated to be depleted with replenishment of the amino acids showing levels of GSH approaching normality after 14 days (Sekhar et al., 2011). Furthermore a pro-
inflammatory response in adipose tissue induced by hyperglycaemia has been shown to be reduced by the introduction of N-acetylcysteine (NAC) (Y. Lin et al., 2005). Interestingly glycine depletion possibly due to glutathione consumption driven by oxidative stress may deplete glycine reflecting increased gluconeogenesis or it could also be indicative of abundant incompletely oxidised fuels that are excreted as urinary acylglycine conjugates (Floegel et al., 2013). The introduction of NAC would therefore provide a reversal role and possibly decrease gluconeogenesis.

A decrease in GSH with a concomitant increase in GSSG has been demonstrated in T2DM patients resulting in a significant decrease in the GSH:GSSG ratio (Calabrese et al., 2012). It has also been found that in patients with impaired fasting glucose (IFG), despite no significant changes in GSH and GSSG levels that the GSH:GSSG ratio was significantly decreased (P=0.04) when compared to a normal control group, suggesting that an impaired redox balance can be evident even when minor increases in blood glucose levels are demonstrable (H.F Jelinek et al., 2013). Decreasing oxidative stress through glycaemic control will therefore potentially be an important mechanism in diminishing the incidence of microvascular complications associated with T2DM.

Antioxidant defense is important in the removal of free radicals, providing the maximal protection of biological sites such as thiol groups which are part of active sites in some metabolizing enzymes (T. Rahman, Hosen, Towhidul Islam, & Uddin Shekhar, 2012). A good antioxidant should specifically quench free radicals, chelate redox metals, interact with other antioxidants within an antioxidant network, have a positive effect on gene expression, be readily absorbed, have a concentration in tissues and biofluids at a physiologically
relevant level, act in both aqueous and/or membrane domains. The most efficient enzymatic antioxidants involve superoxide dismutase, catalase and glutathione peroxidase. Non-enzymatic antioxidants involve thiol antioxidants (glutathione, thioredoxin and lipoic acid), vitamin C, vitamin E, carotenoids, natural flavonoids, melatonin and other compounds (selenium) (K. Rahman, 2007).

1.5.3.2 8-hydroxy-2′-deoxyguanosine

8-hydroxy-2′-deoxyguanosine (8-OHdG) is a product of oxidatively damaged DNA formed by hydroxyl radical, singlet oxygen and direct photodynamic action. 8-OHdG can be detected in tissue, serum, urine and other biological material (Valavanidis, Vlachogianni, & Fiotakis, 2009). Amongst the purine and pyridines the guanine base appears to be the most susceptible to oxidation. It is widely recognised that the most important oxygen free radical causing biomolecular damage is the hydroxyl radical. This radical can be produced by a variety of mechanisms including the Fenton reaction of hydrogen peroxide and metals of other endogenous and exogenous ROS. The interaction of the hydroxyl radical with a nucleobase such as guanine results in the formation of C8-hydroxyguanine or its nucleoside 8-OHdG (Valavanidis et al., 2009). Upon the oxidation of in vivo guanine to the free radical, 8-OHdG is readily excreted into urine by glomerular filtration, thus serving as a useful indicator for the quantification of systemic oxidative stress. Markedly increased levels of urinary 8-OHdG have been shown in T2DM and are positively correlated with HbA1c (Moresco, Sangoi, De Carvalho, Tatsch, & Bochi, 2013) and has been shown to be an early indicator for the development of microvascular and macrovascular
complications in T2DM (Nishikawa et al., 2003). Increased levels of 8-OHdG levels in both prediabetes and diabetes have also revealed statistically significant results upon comparison with cholesterol, MDA and erythrocyte reduced GSH levels, further indicating the utility of 8-OHdG as a potentially useful early marker of vascular damage as a consequence of oxidative stress (Al-Aubaidy & Jelinek, 2010, 2014). This is further confirmed in recent studies conducted by the author (Butkowski et al., 2016) where it was demonstrated that a higher risk of CVD presents with increased 8-OHdG. These findings support previous studies that reflect the antioxidant function of GSH, where the erythrocyte GSH pool decreases at first with increased BGL and CVD risk but due to de novo synthesis in response to continued elevated fasting BGL in the erythrocytes increase (Al-Aubaidy & Jelinek, 2011a; H. F. Jelinek et al., 2014; E.U. Nwose, H.F. Jelinek, R.S. Richards, & P.G. Kerr, 2006, 2007; E. U. Nwose, R. S. Richards, R. G. Kerr, R. Tinley, & H. Jelinek, 2008b).

1.5.3.3 \( F_2 \)- Isoprostanes

The \( F_2 \)- isoprostanes are a group of prostaglandin like compounds formed \textit{in vivo} by the nonenzymatic free radical catalysed peroxidation of non-esterified arachidonic acid. This group of isomers are formed independently from the action of cyclooxygenases (COX-1 and COX-2) (Montuschi, Barnes, & Roberts, 2004). The autoxidation process lacks specificity and leads to the formation of many different structural and stereo-isomers. Isoprostanes, whilst possessing relatively short half-lives, demonstrate potent biological activities, especially in the lungs and kidneys with possible normal physiological functions. (AOC,
Whilst isoprostanes may resemble normal prostanoids the most abundant form is analogous to prostaglandin F2α, while analogues of PGD2 and PGE2 are also found, differing in many aspects of their stereochemistry. Among the sequelae of hyperglycaemia it is widely recognised that excessive oxidative stress is associated with increased vascular disease in DM (Tabak et al., 2011). Early experimental results with thiobarbituric acid reactive substances and lipid hydroperoxides have established the link with atherosclerosis and lipid peroxidation, both within the vasculature and the plasma providing the evidential link between hyperglycaemic induced oxidative stress and diabetic vascular disease (Mezzetti, Cipollone, & Cuccurullo, 2000). More recent evaluations on the utility of isoprostanes has established their accuracy and sensitivity as a useful analytical tool for assessing oxidative stress in a variety of pathologies, along with some isoprostanes to exhibit potent biological activity and thus likely mediators in oxidant injury (L. J. Roberts & Milne, 2009). A meta-analysis of elevated F₂-isoprostanes with CAD, stroke and peripheral artery disease has further consolidated the utility of this biomarker, albeit as a nonspecific indicator of CVD (L. Maschirow, Khalaf, Al-Aubaidy, & Jelinek, 2015; Zhang, 2013). Further detailed ongoing analysis of isoprostane levels may therefore provide useful information as a biomonitor when developing strategies for minimizing vascular complications (Dias & Griffiths, 2014).

1.5.3.4 Malondialdehyde

Reactive oxygen species (ROS) degrade polyunsaturated lipids to form MDA. The aldehyde MDA, as a reactive electrophile species, has the capacity to cause
toxic stress by producing lipid peroxidation end products with the measurement of MDA formation considered to be one of the most frequently quantitative assessments (Nielsen, Mikkelsen, Nielsen, Andersen, & Grandjean, 1997). As lipids are primary targets in the peroxidation the formation of the peroxide radical is considered extremely harmful to the cell membrane. Whilst the actual mechanism of MDA as an endothelial cytotoxic agent is poorly understood it is still considered a useful primary biomarker in the assessment of free radical mediated lipid damage and oxidative stress (Tiwari, Pandey, Abidi, & Rizvi, 2013b). Diabetic studies utilising MDA as a marker of oxidative stress have demonstrated increased levels (E. U. Nwose, H. F. Jelinek, R. S. Richards, & P. G. Kerr, 2007). A recent Veterans Affairs Diabetes Trial had investigated the association with MDA and diabetes and demonstrated that circulating levels of the immune complex MDA – LDL may predict the occurrence of myocardial infarction and acute cardiovascular events in patents with T2DM (Lopes-Virella, Hunt, Baker, Virella, & Moritz, 2012). Interestingly oxidised LDL has been shown to accumulate in atherosclerotic lesions with increasing evidence pointing to a pathogenic role in CAD, ACS and vulnerable plaque. Measurement of MDA has therefore provided further insight into progressive atherosclerosis. Oxidised LDL (oxLDL) has been shown to have suppressive effects on genes regulating endothelial function such as nitrous oxide synthase and also the downregulation of fibroblast growth factor 2 (FG2F). This downregulation is now a recognised form of endothelial dysfunction (Yang et al., 2014).
1.5.3.5 Whole Blood Viscosity

Haemorheology, or blood rheology, is the study of flow properties of blood and its plasma protein constituents. Normal tissue perfusion occurs within certain rheological limits with any alteration contributing significantly to a disease state (Baskurt, 2007). Whilst it is not within the scope of this review to provide a full discussion of whole blood viscosity (WBV), a brief review is warranted due to WBV implications in metabolic disturbances as a result of T2DM or MetS (Gyawali, Richards, Nwose, & Bwititi, 2012). The measurement of WBV is assessed by viscometry, however for routine use it may be calculated via extrapolation using the haematocrit expressed as % and plasma protein level in g/L (Nwose & Richards, 2011).

Any significant increase in WBV has the potential to impair the microvasculature resulting in hyperviscosity syndrome (Kwaan, 2010). Of interest and relevance to this study is the recognition that WBV has an association with developing CVD. The hyperglycaemic state may mediate its adverse effects through a number of metabolic pathways with resultant oxidative stress. The emergent EOS has the capacity to compromise the vasculature (micro and macro). Generated erythrocytic ROS may expose the red cell membrane leading to decrease membrane fluidity and resultant increased aggregation and diminished blood flow (Nwose et al., 2014). WBV is an established phenomena of Virchow’s triad – vascular stasis, endothelial function and atherothrombosis which may ultimately lead to and/or result from CVD complications (Bagot & Arya, 2008). Assessment of WBV and its association with anticoagulant and/or antiplatelet therapy have a positive and negative correlation respectively (Nwose, Cann, &
Butkowski, 2010). In addition an algorithmically derived WBV may be useful in monitoring patients on antiplatelet therapy (aspirin) (Nwose & Butkowski, 2013). Treatment for CVD may incorporate one or both treatment regimens and therefore WBV may be useful as there still is much conjecture on the use of aspirin treatment in T2DM (B. M. Schmidt & Arora, 2013). As more study is required on its efficacy WBV and its association with hypercoagulation, hyperglycaemia, T2DM, CVD will possibly provide more definitive information.

### 1.6 Inflammation in T2DM and CVD

T2DM and CVD are now considered non-communicable chronic diseases with many of them sharing a common inflammatory pathophysiology (Camps & Garcia-Heredia, 2014). This commonality is an inextricable link existing between OS and the inflammatory process and is a common thread throughout this thesis. The following discussion on the implications of the pathophysiological process present in CVD points to the association the pro-inflammatory cytokines and also the expression of adhesion molecules on endothelial surfaces, and the promotion of the initiation of atherogenesis. The inflammatory process stimulates the production of growth factors such as vascular endothelial growth factor (VEGF) and mediates tissue repair with the release of endothelial progenitor cells (EPCs) into the peripheral circulation to participate in tissue repair (C. P. Lin et al., 2013).

This section following reviews the role of both lipids and cytokines in the inflammatory response and provides a review of the cytokines that underwent testing in this thesis.
1.6.1 Inflammation – The lipid connection

The role of lipids in the progress of atherosclerosis is well defined; however the role of the inflammatory process is still subject to some refinement. Enzymatic and free radical oxidation has a prominent role in CVD through their oxidative modification of molecules (Lobo et al., 2010). Lipids are primary targets of this modification because they are a repository of oxidisable double bonds. If this is the case then a link with lipids, OS and inflammation is evident. Dyslipidaemia and the inflammatory response are considered causal risk factors in the development of atherosclerosis (van Diepen, Berbee, Havekes, & Rensen, 2013) and there appears to be an association with IL-1β and obesity induced inflammation and the development of insulin resistance in adipose tissue (Stienstra et al., 2010).

More recent additions to the family of inflammatory and regulatory and lipid markers to come under scrutiny is phospholipid oxidation products (McIntyre & Hazen, 2010). The elucidation of the emerging phospholipid oxidation products associated with the family of inflammatory and regulatory mediation is currently topical and under investigation (Kim, 2015). The relationship of dyslipidaemia, hyperglycaemia and the acute phase response is discussed further at 1.6.3.

1.6.2 Inflammation and hyperglycaemic states

Non-insulin-dependent diabetes mellitus (NIDDM) or T2DM is commonly associated with hypertriglyceridemia, low serum HDL-cholesterol
concentrations, hypertension, obesity and accelerated atherosclerosis. Emerging literature in the 1990’s viewed inflammatory processes as a component of atherothrombosis with some skepticism with discussion premised upon bench observations (Ridker, 2010). By 2000 a plethora of literature had emerged *inter alia* that atherosclerosis was the product of an inflammatory process (Ridker, Hennekens, Buring, & Rifai, 2000).

MetS is considered to be a major classification, diagnostic and therapeutic challenge as hyperglycaemia may or may not be present in patients demonstrating hypertension, central upper body obesity and dyslipidaemia. MetS never-the-less presents as a high risk group for the development of macrovascular disease (K. G. Alberti & Zimmet, 1998). Some confusion still exists in the classification of MetS with the constellation of risk factors varying and dependent upon the organisation that proposed them (Kassi, Pervanidou, Kaltsas, & Chrousos, 2011). The National Cholesterol Education Program’s Treatment Panel report (ATPIII) consolidated the collaborative work of the American Heart Association and the National Heart, Lung, and Blood Institute has identified MetS (see Table 6 for ATP III list) (S. M. Grundy, H. B. Brewer, J. I. Cleeman, S. C. Smith, & C. Lenfant, 2004). Extended criteria for CVD risk and metabolic syndrome have since been proposed and are tabulated with ATP III in Table 6.
### TABLE 6. ATP III MetS criteria and extended CVD risk

<table>
<thead>
<tr>
<th>ATP III MS criteria</th>
<th>Extended criteria for CVD risk</th>
<th>Additional indicia related to CVD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central obesity</td>
<td>Major clinical outcomes</td>
<td>Atherogenic dyslipidaemia</td>
</tr>
<tr>
<td>Elevated triglycerides</td>
<td>Metabolic components</td>
<td>Proinflammatory state</td>
</tr>
<tr>
<td>Low HDL</td>
<td>Pathogenesis</td>
<td>Prothrombotic state</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Clinical criteria for diagnosis</td>
<td>Insulin resistance +/- glucose intolerance</td>
</tr>
<tr>
<td>Hyperglycaemia</td>
<td>Risk for clinical outcomes</td>
<td>Therapeutic interventions</td>
</tr>
</tbody>
</table>

These combinations identified as a result of the “obesity epidemic” can be deemed a major contributory factor in the emergence of MetS (Scott M. Grundy et al., 2004).

1.6.3 Dyslipidaemia and acute phase response

There is recognition that a similar dyslipidaemia may occur with an acute-phase response to external stimuli. Pickup et al (1997) investigated as to whether elevated acute-phase/stress reactants (the innate immune system's response to environmental stress) and their major cytokine mediator interleukin-6 (IL-6) were associated with NIDDM and MetS. Part of this investigation was to attempt to provide a unifying pathophysiological mechanism for these two conditions. Pickup et al conducted a study of two groups of Caucasian subjects with NIDDM. The cohort with any 4 or 5 features of Metabolic syndrome MetS (n = 19) were compared to a group with 0 or 1 feature of MetS (n = 25), but with similar age, sex distribution, diabetes duration, glycaemic control and diabetes treatment. Healthy non-diabetic subjects of comparable age and sex acted as controls.
Overnight urinary albumin excretion rate, a predictor risk factor for cardiovascular disease, was also assayed in subjects to assess its relationship to the acute-phase response. Serum sialic acid was confirmed as a marker of the acute-phase response since serum concentrations were significantly related to established acute-phase proteins such as alpha-1 acid glycoprotein (r = 0.82, p < 0.0001). There was a significant graded increase of serum sialic acid, alpha-1 acid glycoprotein, IL-6 and urinary albumin excretion rate amongst the three groups, with the lowest levels in non-diabetic subjects, intermediate levels in NIDDM patients without MetS and highest levels in NIDDM patients with MS. C-reactive protein and cortisol levels were also higher in MetS-positive compared to MetS negative patients and serum amyloid A was higher in both diabetic groups than in the control group. It was concluded that NIDDM is associated with an elevated acute-phase response, particularly in those with features of MetS. Abnormalities of the innate immune system may therefore be considered to be a contributor to the hypertriglyceridaemia, low HDL cholesterol, hypertension, glucose intolerance, insulin resistance and accelerated atherosclerosis of NIDDM. It could also be concluded that microalbuminuria is a component of the acute-phase response (Pickup, Mattock, Chusney, & Burt, 1997). Microalbuminuria is a proven marker for assessment in T2DM patients and any progression through to renal nephropathy. As a marker for CVD predictability, however the findings are not conclusive.

1.6.4 Inflammation and the plaque formation process

Inflammation contributes too many of the characteristics of plaques implicated in the pathogenesis of CVD and possible resultant acute coronary syndrome. The
inflammatory process may not only regulate the properties of plaques but also modulate the clinical consequences of the thrombotic complications of atherosclerosis. Hyperglycaemia and the formation of advanced glycation end products (AGE) along with AGE modified LDL has effects on the vascular wall but has been associated with insulin resistance even with and without evidence of the presence of DM. The association of AGE – LDL has been shown to directly affect the vascular wall and induce gene expression. Exposed THP-1 cells to AGE- LDL have been shown to increase MCP-1 threefold when compared to the cells which underwent non AGE – LDL (Isoda, Folco, Marwali, Ohsuzu, & Libby, 2008; Panee, 2012); thus highlighting that the progression to diabetic atherosclerosis assumes complex interactions when considering biological modifications to LDL. Interestingly AGE products have been reported to increase ROS formation and impair antioxidant systems, whereas some AGE product formation results from increased oxidative stress (Nowotny, Jung, Höhn, Weber, & Grune, 2015). There is general acceptance that inflammation and dyslipidaemia, irrespective of the initiator, participate in the formation of the atheroma (Manduteanu & Simionescu, 2012). Figure 5 provides a pictorial summation of the atherosclerotic changes and associated cytokine interactions. While not totally dissimilar to the cartoon I propose (Fig.1) the detail in Fig. 5 does not incorporate all of the specific OS and inflammatory biomarkers but does provide a good overview of the thrombus formation.
The altered hydrodynamics (also see discussion on whole blood viscosity) causes a loss of the athero-protective functionality of the endothelial cells including the vasodilator, anti-inflammatory, fibrinolytic and anticoagulant properties. Antigens presented to antigen presenting cells (APC) such as dendritic cells can activate T helper (TH1) lymphocytes to produce interferon – γ (IFN-γ) which in turn activates macrophages. Other lymphocytic subtypes, including TH2 cells can elaborate the anti-inflammatory cytokine interleukin-10 (IL-10) and regulatory T cell and regulatory T cells that secrete the anti-inflammatory cytokine transforming growth factor-β (TGF-β). The macrophage surface contains Toll-like receptors (TLRs) 2 and 4, which have the ability to bind pathogen-associated molecular patterns and damage-associated molecular
patterns (B. A. Beutler, 2009; Kawai & Akira, 2011). The intracellular TLRs 3, 7, and 9 may also contribute to lipid accumulation and other proatherogenic functions of the macrophage. Macrophages can undergo stress of the endoplasmic reticulum (ER) under artherogenic conditions. Within the plaque cholesterol crystals also may activate inflammatory nucleotide-binding oligomerisation domain (NOD), leucine-rich repeats (LRR) and pyrin domain-containing three inflammasome that can generate mature IL-1β from its inactive precursor (P. Libby et al., 2014; Sheedy et al., 2013). The support to the plaque’s fibrous cap by the triple helical interstitial collagen may undergo degradation by collagenase secreted by macrophages. Tissue factor, potent procoagulant and proinflammatory cytokines elicited by macrophages have a tendency to amplify and sustain the inflammatory process within the plaque (P. Libby et al., 2014). The activated macrophage secretes collagenases that can degrade the triple helical interstitial collagen that lends strength to the plaque’s fibrous cap.

1.6.5 Stress and inflammation

Repeated episodes of acute or chronic psychological stress can induce an acute phase response (APR) and therefore contribute to a chronic inflammatory process such as atherosclerosis (Black & Garbutt, 2002). The stress process may be extended to include a chronic inflammatory process(s), characterised by the presence of certain cytokines and APR’s associated with certain metabolic diseases. The site of origin and of the cytokines, of origin of these cytokines, particularly IL-6 and its induction is likely the liver, endothelium, and fat cell depots. It is probable that CRP, is associated with and plays a dominant role in,
the development of this inflammatory process and contributes to insulin resistance, MetS and T2DM (Black, 2003). The role of psychological stress and the major stress-related hormones as etiologic factors in the pathogenesis of these metabolic diseases, as well as atherosclerosis, may also need to be considered when assessing or evaluating biomarkers (Ranabir & Reetu, 2011). The fact that stress can activate an APR, which is part of the innate immune inflammatory response, is evidence that the inflammatory response is contained within the stress response, or that stress can induce an inflammatory response. (Black, 2003).

Chronic inflammation associated with the metabolic and immune systems involves a network of both cellular and systemic responses integrating a complex signaling pathway mediated by major stress hormones: noradrenaline and adrenaline, and cortisol; angiotensin II and proinflammatory cytokines. These may enter the circulation as a product of lipolysis from adipose tissue (Lamb & Goldstein, 2008).

However, it is not the role of this discussion to attempt to review all stress related factors. Issues which do become evident are the interactions between many of the biomarkers reviewed and the role they may assist in the formation and predisposition of patients developing T2DM and/or CVD.

1.7 Inflammatory biomarkers

The inflammatory process is a second tier natural physiological reaction and defense mechanism occurring in response to any insult upon tissue or organs considered foreign to the organism. The response is diverse as it is predictable. There is wide recognition that in T2DM and CVD, just as oxidative stress is
implicated so is the inflammatory process. Traditional and emerging biomarkers considered to be contributory to this process are further discussed. The importance of the obese state as a contributor to the inflammatory process cannot be underestimated. Adipose (fat) tissue, consists of adipocytes and other cell types embedded in loose connective tissue. Adipose tissue was originally thought of as an energy storage repository, however it is now recognised that adipocytes release, amongst other things, adipokines a group of bioactive mediators. These substances are known to modulate haemostasis, BP, lipid and glucose metabolism, inflammation and atherosclerosis (Rabe, Lehrke, Parhofer, & Broedl, 2008). With obesity a dysregulated secretion of a number of pro-inflammatory cytokines such as adinopectin and IL-10 and anti-inflammatory cytokines including IL-1, IL-1β, IL-6, MCP-1, TNF-α, TGF-β, leptin, resistin, angiotensin occurs (Jung & Choi, 2014). Whilst many of these cytokines are not exhaustive or exclusive to only adipose tissue, the inflammatory cytokines of relevance to this thesis are discussed.

1.7.1 C Reactive Protein

The inflammatory marker and acute phase reactant protein CRP could be considered a surrogate marker for assessing cardiovascular risk. CRP was discovered in 1930 and received its name by virtue of its reaction with the C-polysaccharide of pneumococcus in the acute phase of pneumococcal pneumonia (Aguiar et al., 2013). The physiological function of CRP is to induce a non-specific defense mechanism against infection and to assist macrophages to scavenge altered lipoproteins.
The use of high sensitive CRP (hsCRP) as a potential marker for CVD is premised upon its role in inflammation as an infective process in CVD. It atherosclerotic plaques increased numbers of monocytes and T cells attracted by deposits of oxidised LDL’s accumulate in the arterial wall (Paffen & deMaat, 2006). This is evidenced by the transformation of monocytes to macrophages that commence the ingestion of cholesterol; the excretion of cytokines, growth factors and adhesion factors (IL 1 and 2, TNFα, interferon γ and CSF). In addition acute phase reactants such as CRP, fibrinogen and Interleukin 6 (IL-6) may be elevated in atherosclerotic patients. An increase in IL-6 in turn will stimulate the hepatic production of CRP (Bock, 2007). The development of the higher sensitivity CRP assays for routine testing has afforded a tightening of the reference range, currently quoted at: 0 – 3.0 mg/L. CRP, as an acute phase protein has long been established as a robust predictor of the inflammatory response. It is not directly involved in any coagulative process and is considered a sensitive objective marker of inflammation, tissue damage and infection. In contrast to virtually all other major acute phase reactants and the coagulation proteins, CRP’s half-life (19 hours) is rapid and remains constant under all conditions making its synthetic rate the sole determinant of its plasma concentration (Koenig et al., 1999). Interestingly growing evidence indicates that elevated circulating inflammatory markers in acute coronary syndrome (ACS), in particular CRP, is predictive of an unfavourable course, independent of the severity of atherosclerotic or ischaemic burden (Peter Libby, Ridker, & Maseri, 2002). Studies by Arnalich et al. on whether oxidative stress could promote a systematic acute-phase response in elderly patients with T2DM have found that multiple regression analysis of markers such as lipid peroxidase and IL-6 correlated independently with CRP;
suggesting oxidative stress might be implicated in promoting a state of low-grade systemic inflammation in this patient cohort (Arnalich et al., 2000). Further, modelling studies in animals have demonstrated that antagonists of the inflammatory cytokine pathway appear to block mononuclear cell binding to arterial plaque, possibly indicating CRP may play a proinflammatory role in activating monocyte chemotactic (chemoattractant) protein. Anti-atherosclerotic medication may therefore exert some beneficial effect by inhibiting harmful effects attributed to CRP (Yeh, 2004). As a relatively nonspecific and inexpensive biochemical marker for the inflammatory process multivariate correlation with a marker more predictive of pre DM and/or CVD could be deemed very useful in preventative medicine. Further work continues on the elucidation of the mechanism for CRP and atherosclerosis as evidence demonstrates that CRP may modulate the expression of genes that contribute to both pro- and anti-inflammatory responses in human monocytes. Among the anti-inflammatory effects, CRP may also activate the Liver X receptor α (LXRα) pathway (Hanriot et al., 2008). Interestingly, studies utilising dihydrocapsaicin, an analog and congener of capsaicin fed to Apo-E−/− mice has the potential to inhibit atherosclerotic plaque formation. A number of inflammatory markers such as IL-1β, IL-6 and CRP were shown to be decreased (Hu et al., 2013), thus providing further evidence on the contribution of inflammation on the atherosclerotic plaque formation.
1.7.2 Complement 5a

C5a is an anaphylotoxin protein derived from the complement system. C5a and C5b are released from the cleavage component of C5 by a protease C5-convertase. Whilst C5b is important in the late events of the activated complement cascade C5a acts as a highly inflammatory peptide with chemotactic and anaphylotoxin functionality. C5 originates from the hepatocyte but synthesis can also be demonstrated in macrophages, thus providing a localised response to inflammation (Ramadori, Rasokat, Burger, Meyer Zum Buschenfelde, & Bitter-Suermann, 1984). It therefore provides a function in the innate immunity but also has a role in the adaptive response. C5a has been shown to demonstrate potent chemotactic and proinflammatory effects in atherosclerotic lesions and many other inflammatory conditions. Whilst complement activation is indicated by increased levels of C5a in CVD more data and investigation is required to establish the utility of C5a as a useful predictable biomarker (Speidl et al., 2005). It has been demonstrated that the C5a receptor CD88 is present on many atherosclerotic cells within plaques thus implicating C5a as a pro inflammatory mediator. Studies on the arteries of non-fat fed female ApoE⁻/⁻ mice expressing C5a and CD88 receptors demonstrated a reduction in plaque size following PMX53 CD88 receptor antagonist treatment (Manthey et al., 2011). This study therefore may provide evidence that C5a levels may be of use as a predictor of CVD, however further studies are required for elucidation.
1.7.3 The interleukins

The interleukins, a large group of cytokines, gained their nomenclature from their discovery of expression in white blood cells; however the naming is somewhat of a relic as many cells are now known to produce these cytokines. In general the term ‘Interleukin’ is now used to describe a group of cytokines which possess complex immunomodulatory functions inclusive of cell proliferation, maturation, migration and adhesion (Brocker, Thompson, Matsumoto, Nebert, & Vasiliiou, 2010). Therefore the functionality of the immune system is very dependent upon these mediators.

1.7.3.1 Interleukin - 1β

IL-1α and IL-1β are known to participate in the regulation of the immune process, inflammation and haematopoiesis, exerting their proinflammatory pleiotropic effects on a variety of cells that participate in acute and chronic inflammatory and autoimmune disorders (Ren & Torres, 2009).

In vitro, IL-1β-mediated auto-inflammatory process results in beta-cell death. The auto-inflammation is driven by glucose, free fatty acids, leptin, and IL-1β itself. Caspase-1 is required for IL-1β activity and the release of free fatty acids from the adipocyte. Patients with T2DM have demonstrated an imbalance with the IL-1β agonist activity versus specific countering by the naturally occurring IL-1 receptor antagonist(IL-1Ra) determining islet inflammation outcome (Dinarello, Donath, & Mandrup-Poulsen, 2010). Whilst it is recognised that the pro-inflammatory nature of IL-1β in a variety of what are considered auto-inflammatory conditions anti IL-1β treatments such as the natural occurring IL-
1 receptor antagonist – Anakinra and anti-IL-1β monoclonal antibodies have been shown to improve glycaemic control (Dinarello, 2011). Further investigation of this biomarker as a predictor would tend to rely upon the observable levels obtained and its utility. The propensity for an in vivo auto-inflammatory process may therefore require further genetic studies and receptor analysis.

1.7.3.2 Interleukin – 6

Interleukin 6 (IL-6) is secreted to stimulate an immune response during infection, or tissue damage leading to the inflammatory response (APR) and acts as a haematopoietic agent (Kleemann, Zadelaar, & Kooistra, 2008). IL-6 can also be released by adipose and skeletal muscle and is considered to have both an inflammatory and anti-inflammatory action and as a systemic adipokine is shown to impair insulin sensitivity and also aids in the hepatic production of the APR protein CRP (Kaur, 2014b). High levels have been associated with obesity and insulin resistance with a direct correlation with increased incidence of CHD, however limited studies have seen an association with vascular complications in T2DM (G. Lowe et al., 2014). A recent study by the author found a significant association with IL-6 and T2DM, but failed to show significance with CVD risk (Butkowski et al., 2016). Further studies with a larger sample size is required to verify these findings.

IL-6 cytokine is also implicated in the regulation of metabolic, regenerative and neural processes and in classic signaling targets cells via a membrane bound receptor and associates with the signaling receptor protein gp130 and subsequent
activation of mitogen-activated protein (MAP) (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). Numerous studies have identified that IL-6 will induce the CRP gene in hepatocytes enhancing the increased production of the inflammatory marker. Regulation of the acute phase response (APR) is mediated by the inflammatory cytokines IL-6, TNF-α and IL-1β either acting alone, together or in combination with steroid hormones resulting in the up regulation of the CRP and serum amyloid P-component (SAP) genes (Szalai, van Ginckel, Wang, McGhee, & Volanakis, 2000). Wang has depicted a relationship with obesity, inflammation and CVD. With ensuing obesity the hypertrophy of adipocytes results in molecular changes and cellular alterations affecting systemic metabolism. The accumulation of macrophages within the adipose tissue leads to a local inflammatory response. The expression of several pro-inflammatory mediators occurs in adipocytes including IL-6 and TNF-α sustains vascular wall inflammation and promotes pro-atherogenic gene expression (Z. Wang & Nakayama, 2010). IL-6 and TNF-α are also responsive to exercise stress with levels shown to be increased up to 100 fold in marathon runners (Bente Klarlund Pedersen, 2000), however as levels may increase proportionally, dependent upon the degree of exercise intensity, it would be appropriate to consider full activity history when reviewing the marker as an indicator for CVD and T2DM, due to the likelihood of increased levels associated with exercise. It has been further demonstrated that significant increases in IL-6 levels post exercise are apparent (Al-Aubaidy & Jelinek, 2010), confirming caution is required when interpreting this inflammatory marker. The fact that IL-6 has both pro and anti-inflammatory mechanisms highlights the caution required in interpreting results for inflammation alone. Considering this biomarker in
conjunction with high sensitivity CRP, either correlated or as a ratio should be further investigated for clarification of any role which gauges the inflammatory response to occur in MetS, prediabetes, T2DM and its association with CVD.

1.7.3.3 Interleukin - 10

Interleukin 10 (IL-10) functions as a pleiotropic cytokine primarily effecting inflammation and immunoregulation. It down regulates the expression of T helper 2 cytokines, MHC class II antigens and co-stimulatory molecules on macrophages (Baidya & Zeng, 2005) and is considered one of the main anti-inflammatory interleukins (García-Moll, 2005; Seruga, Zhang, Bernstein, & Tannock, 2008). Its potent anti-inflammatory properties play a central role in limiting host immune responses to pathogens and autoimmune pathologies. IL-10 further has regulatory and growth and/or differentiation effects on a variety of haematopoietic cells including: B cells NK cells, cytotoxic and T helper cells, mast cells, granulocytes, dendritic cells, keratinocytes and endothelial cells (K. W. Moore, de Waal Malefyt, Coffman, & O’Garra, 2001).

As an anti-inflammatory agent IL-10 may well have anti atherogenic properties as demonstrated in mice models that have over expression of IL-10, or alternatively in excessive plaque formation in IL-10 deficient atherosclerotic prone mice (Han & Boisvert, 2015; Han, Kitamoto, Wang, & Boisvert, 2010). A positive association of IL-10 in elderly without a history of CVD events appear to provide some merit as a biomarker however this association is weaker with a prior history of CVD (Welsh et al., 2011). The question will still remain on how IL-10 may be utilised to detect pro-inflammatory processes in CVD. A study
utilising biomarkers on obese, MetS and DM patients has demonstrated significantly increased levels of IL-10 (Fernández-Bergés et al., 2014) however the current paucity on IL-10 as a predictor of DM and CVD requires further investigation. The efficacy of interpreting IL-10 results as a standalone predictor for T2DM and CVD should proceed cautiously as its ubiquitous actions inclusive of its widespread pleiotropic effects, suppression of immune responses and its involvement in a wide range of immunological disorders, Th 1 and Th 2 hypersensitivities along with enhanced regulatory responses due to improper clearance of pathogens and tumour cells (Bijjiga & Martino, 2013) should invite discretion pending further investigation.

1.7.3.4 Monocyte Chemoattractant Protein - 1

The chemokine ligand 2 (CCL2) also referred to as: MCP-1 is responsible for the recruitment of monocytes, memory T cells and dendritic cells to inflammatory sites and induce chemotaxis through the activation of G-protein-coupled receptors. This cytokine is produced by a variety of cell types, including monocytes, fibroblasts, smooth muscle, endothelial, mesangial, astrocytic and microglial cells. However, a primary source of MCP 1 originates within the monocyte/macrophage series which in turn provide infiltration and migration regulation of monocytes, memory cells and NK cells (Deshmane, Kremlev, Amini, & Sawaya, 2009). Higher levels of MCP-1 have been demonstrated in atherosclerotic patients undergoing hemodialysis (Kusano et al., 2004). Atherogenesis and the role MCP-1 contributes have been tested in LDL receptor deficient mice which have been genetically modified to be MCP-1 deficient.
These mice were fed a high cholesterol diet demonstrating 83% less lipid accumulation in the aorta and less macrophage presence indicating the important role MCP-1 may contribute to atherogenesis (Van Coillie, Van Damme, & Opdenakker, 1999).

Murine studies have also demonstrated that insulin has induced the in vitro expression of MCP-1 in insulin resistant 3T3-L1 murine adipocytes and in vivo in IR obese mice. Experimental data has indicated that MCP-1 (and mRNA) appears to have promoted insulin resistance in differentiated adipocytes along with the down-regulated expression of adipogenic genes including LpL, adipsin, GLUT-4, aP2, 3-adrenergic receptor and PPAR-γ (Sartipy & Loskutoff, 2003).

Diabetic patients with nephropathy also show increased levels of MCP-1, transforming growth factor beta-1(TGF-β1), connective tissue growth factor (CTGF) and fibronectin (FN) (El Mesallamy, Ahmed, Bassyouni, & Ahmed, 2012), providing evidence for the inflammatory association of MCP-1 with the diabetic state.

A recent review on MCP-1 has inter alia provided a broad ranging discussion contributing to our current understanding on how MCP-1 contributes to the development of obesity, DM, CVD, insulinaemia, diabetic nephropathy and retinopathy (Panee, 2012). Whilst not within the scope of this review the significance of MCP-1 and its pathophysiological contribution too many of the biochemical pathways cannot be understated. This may well include the association of MCP-1 with the hyperglycaemic state and AGE products. The prevalence and deleterious effects of AGE and the reduced functionality of endothelial cells through the reduced production of NO noting its anti-inflammatory nature provides further evidence to the importance of comparative
analysis of various biomarkers (Wautier & Schmidt, 2004). Isoda et al provide further characterisation, although incomplete, on the pathophysiological effects of AGE-LDL and the stimulated proliferation and differentiation of vascular smooth muscle and the increased expression of the MCP-1 receptor on endothelial cells (Isoda et al., 2008). AGE products have also been associated with insulin resistance even in the absence of the presence of DM. AGE may exert their pathogenic effects by interaction with a variety of cell surface receptors, namely RAGE (receptor for AGE), CD36, lectin oxidised LDL – R1, macrophage scavenger receptors contributing to AGE formation and accumulation. The signaling of AGE – RAGE results in increased ROS primarily through the nicotinamide adenine dinucleotide phosphate – oxidase system (Song & Schmidt, 2012).

1.8 Insulin like growth factor - 1

Whilst IGF-1 may not be considered an inflammatory factor per se its role in the development of cell growth and differentiation and tissue repair along with potential mechanisms for atherogenic protection has attracted a great deal of interest. Potentially this protection interacts with oxidative stress, pro-inflammatory signaling and cell apoptosis (Higashi, Sukhanov, Anwar, Shai, & Delafontaine, 2012).

IGF-1 (Somatomedin C) is a 70-amino acid - basic polypeptide contributing a fundamental role as a major postnatal potent growth mediator for hormone activity (Rotwein, Pollock, Didier, & Krivi, 1986). Both IGF-1 (and IGF-2) ligands show some structural homology to proinsulin (Rajpathak et al., 2009),
relaxin and β-nerve growth factor (β-NGF) (Hoppener et al., 1985). This growth promoting peptide is secreted by a variety of cells and possesses a multifunctional action. This action primarily stimulates the proliferation and differentiation of many cell types eliciting its effects by binding to its putative receptor (IGF-1R). IGF-1 may be produced in the liver in response to growth hormone (GH) stimulus (Smith, 2010) however, virtually any cell may secrete for autocrine or paracrine purposes. Coherency in the function of IGF-1 is undergoing constant clarification due to its ubiquitous prevalence and association with numerous pathologies. IGF-1 deficiencies are prevalent in a diverse array of conditions and pathologies (Puche & Castilla-Cortazar, 2012).

Circulatory transport of IGF-1 occurs via the binding proteins (IGFBP), of which to date six of these binding proteins have been cloned and sequenced (Hwa, Oh, & Rosenfeld, 1999). The jury may still be out in relation to the utility of IGF-1 as a predictor biomarker for CVD and T2DM, as there are possible associated complex dynamics of the IGFBP providing modulation of IGF activity (Schneider, Wolf, Hoeflich, & Lahm, 2002); along with implications that IGFBP related protein 1 (IGFBP-rP1) may also interact with the IGF system (López-Bermejo et al., 2006).

Studies on levels of IGF-1 in DM are therefore variable. Teppala when examining diabetic patients by age category noted lower levels of IGF-1 having a positive association with patients <65yrs of age, whereas this was not the case in >65yrs of age (Teppala & Shankar, 2010). This evidence has supported previous findings where low levels of IGF-1 and glucose intolerance/diabetes provide for a positive association, further supporting evidence of the possible important interaction of IGF-1 and IGFBP and glucose homeostasis (M. S.
Low levels of IGFBP-1 and raised levels of CRP have been associated with increased risk of MetS (Kaushal et al., 2004).

Recent studies reviewing the actions of IGF-1 and the ageing process, and the inverse relationship of lower levels of IGF-1 associated with increased risk of atherosclerosis and vascular disease are highly suggestive of the anti-inflammatory effects of IGF-1 (Higashi et al., 2012). Low levels of IGF-1 are well documented in ensuing cardiovascular and cerebrovascular disease (Ungvari & Csiszar, 2012). Prospective studies on lower levels and its association with the development of coronary artery disease and as a predictor of fatal ischaemic heart disease has been demonstrated (Juul, Scheike, Davidsen, Gyllenborg, & Jørgensen, 2002; Laughlin, Barrett-Connor, Criqui, & Kritz-Silverstein, 2004), with lower levels also predictive of poorer outcomes in myocardial infarction (E. Conti et al., 2001). Indirect evidence of the atherogenic effects of IGF-1 point towards its ability to induce an in vivo proliferation of vascular smooth muscle however a further explanation may reside in the ability to initiate a survival pathway aimed at compensating local vascular cell apoptosis, possibly by directly opposing endothelial dysfunction either increasing nitric oxide levels through high affinity endothelial binding sites with IGF-1, the promotion of insulin sensitivity and potassium channel opening and the prevention of post prandial dyslipidaemia (Elena Conti et al., 2004). The potential plaque stabilizing role through its anti-apoptotic effects on smooth muscle cells therefore does require further elucidation (von der Thüsen et al., 2011).

Primary or secondary deficiencies of IGF-1 have been associated with shortened life span (Laron, 2008) with many drug agencies (legal and questionable) touting the miracle hormone as the fountain of youth. The corollary however is the
protection afforded to congenital deficiencies of IGF-1 and possible protection to the post-natal development of malignancies (Steuerman, Shevah, & Laron, 2011). Either way low levels of IGF-1 and the increased risk to T2DM does require further consideration and categorisation on its association with its physiological effects on glucose metabolism (Thankamony et al., 2014).

1.9 Hyperglycaemia and coagulability

In unravelling the pathophysiological processes T2DM increased incidences of atherothrombotic and venous thrombosis occur. Oxidative stress and diminished levels of nitrous oxide (NO) result from the impairment of endothelial nitric oxide synthase; all of which contribute to the pro-atherogenic state (Tousoulis et al., 2013). Hypercoagubility and hypofibrinolytic impairment are evident in T2DM (Lemkes et al., 2010). In essence the underlying mechanisms for the increased likelihood of thrombotic events in T2DM is multifactorial with premature atherosclerotic lesions predisposing subjects to plaque rupture and thrombus (Fig 3). Endothelial dysfunction as a consequence of the impaired balance between coagulation and fibrinolysis, platelet activation along with architectural changes to the smooth muscle proliferation within the vasculature all contribute to the increased risk of thrombotic episodes (Alzahrani & Ajjan, 2010; L. Maschirow et al., 2015).

Recognition of the interaction between the oxidative stress state, inflammatory response and the interaction with both the hypercoagulable and fibrinolytic process invites the consideration of markers contributing to hemostasis.
1.9.1 Thrombus formation

With the formation of a thrombus (see Fig’s 1 and 4) polymorphonuclear leukocytes (PMNs) can accumulate and release myeloperoxidase (MPO) and the potent pro-oxidant hypochlorous acid (HOCl) (Eiserich et al., 1998). Dying PMNs extrude DNA that can form neutrophil extracellular traps, entrap leukocytes and propagate thrombosis. Other inflammatory cells modulate atherosclerosis. B1 lymphocytes secrete natural antibody that can inhibit plaque inflammation, however, B2 lymphocytes in part via B-cell activating factor (BAFF), can promote inflammation and plaque complication. Mast cells will also augment atherogenesis by releasing histamine and the cytokines IFN-γ and IL-6 (P. Libby et al., 2014). In addition it is recognised that adipose tissue may produce TNF-α and IL-6, therefore obesity will possibly increase the prospect of the inflammatory response and potentiate atherogenesis, independent of insulin resistance or lipoprotein effects (Peter Libby et al., 2002).

Systemic inflammation can give rise to cytokines, culminating in the overproduction of IL-6, the trigger of the hepatic acute phase response. The acute phase reactant (APR) fibrinogen will then participate directly in thrombus formation. Another acute phase reactant, plasminogen activator inhibitor-1 (PAI-1), can impair fibrinolysis by inhibiting the endogenous fibrinolytic mediators, urokinase- and tissue-type plasminogen activators (uPA and tPA) (P. Libby et al., 2014).

Fibrinogen itself has associations with CVD but as an APR elevated results should be cautiously interpreted, however other coagulation markers such as D-
dimer, C5a (considered pro-inflammatory and coagulative) and tPA have been associated with CVD risk (G. Lowe & Rumley, 2014; L. Maschirow et al., 2015) It is therefore also important to consider the coagulative state and review associated markers as there is a close association between the coagulative and oxidative processes.

1.9.1.2 Coagulative and fibrinolytic markers

Atherothrombotic episodes are the sequelae of complications arising in subjects with DM. The thrombosis, due to plaque disruption, is considered part of the end stage progression of atherosclerosis. In essence the coagulative and hypofibrinolytic state which is known to be a main cause of mortality in diabetic patients can underestimate the importance of our understanding of the third part of our biomarker narrative. Following is a discussion of the coagulation markers which may provide insight into the relationship and interaction that occurs with the oxidative stress and inflammatory markers. Homocysteine is incorporated in the coagulative discussion, however there is a recognised link between elevated levels of homocysteine, inflammation and hypercoagulability and increased CVD risk (Aso et al., 2004).

1.9.1.3 Homocysteine

Homocysteine (Hcy) is a sulphur containing amino acid that does not incorporate into proteins but exists as a metabolic intermediate (Blom & Smulders, 2011). The molecule may either be methylated to form methionine or proceed through the transsulphuration pathway to cystathione and then to cysteine. Hcy can exist
in the plasma as the species with a free sulphydryl group as the disulphide homocysteine or as a mixed disulphide linked to a protein via a cysteine residue. The sum of these are referred to as total Homocysteine (tHcy), however for assay purposes when estimating plasma levels it is simply referred to as Hcy (Bock, 2007).

Elevated levels of Hcy have long been known as having deleterious effects on endothelial or smooth muscle cells, with mild elevations demonstrated in vascular disease. Animal modelling has indicated that the effects of elevated levels of Hcy is multifactorial, affecting both the vascular wall structure and coagulation (Selhub, 1999). Beyond thrombosis, impairment of endothelium-dependent vasorelaxation, and proliferation of vascular smooth muscle cells, Hcy can also promote the synthesis of proinflammatory mediators. Whilst elevated levels of Hcy and CRP may explain some of the biochemical mechanisms in disease progression, the increased plasma concentrations and the inflammatory processes associated with the pathogenicity of atherothrombotic disease remains incompletely understood. Most hypotheses suggest that Hcy contributes to enhanced vascular inflammation in part via oxidative stress (Olszewski & McCully, 1993). It is possible that the thiol group of Hcy undergoes autoxidation in plasma to generate ROS which has the capacity then to contribute to endothelial cell injury or dysfunction (Ventura et al., 2009). The mechanism to associate the dysfunction of vascular dysfunction and elevated levels of Hcy may be a resultant depletion of bioavailable nitrous oxide (Maron & Loscalzo, 2006).

Aetiological factors for atherosclerosis are believed to increase the conversion of methionine to the reactive cyclic lactone homocysteine thiolactone. The free amino groups of LDL are then thiolated causing increased uptake by
macrophages of aggregated LDL (a key factor step in foam cell formation), explaining lipid deposition in atheromas. The release of the Hcy thiolactone from the LDL complex within the vascular wall promotes intimal injury, oxidation of cholesterol and unsaturated lipids, platelet aggregation, thrombogenic factors, myointimal hyperplasia, deposition of glycosaminoglycans, fibrosis and calcification in atherosclerotic plaques (McCully, 1993). An interesting component of the implications of oxidative stress as proposed by Ventura et al. is its action as a potential mediator in the aging process paralleling the increased activity of NADPH oxidase, a main contributor to the production of superoxide anion as reported in atherosclerotic lesions. It is possible that NADPH oxidase could be activated by Hcy in addition as well as inflammatory mediators such as cytokines or CRP. Further the interactions between endothelial cells and monocytes promoted by Hcy or inflammation could enhance NADP oxidase activity (Ventura et al., 2009). In addition to the proinflammatory and coagulative component of Hcy it is also now known to promote insulin resistance (Eguchi & Manabe, 2013).

1.9.1.4 D-Dimer

D Dimer, a high molecular weight fibrinogen derivative originates from cross linked D fragments of fibrin. It is reflective of both thrombin formation and the activation of fibrinolysis and has also been implicated as an inflammatory marker (Spronk, van der Voort, & Ten Cate, 2004). Upon the cleavage of thrombin a cryptic polymerization site on fibrinogen is exposed which promotes the binding of either additional fibrinogen or monomeric fibrin. Protofibrils are
formed from overlapping fibrin monomers which are then covalently crosslinked under the auspices of factor X111a producing the fibrin degradation products (Adam, Key, & Greenberg, 2009). Plasmin, a serine protease degrades fibrin releasing these fibrin degradation products, exposing the D-dimer antigen epitope (Gaffney, Edgell, Creighton-Kempsford, Wheeler, & Tarelli, 1995). These higher molecular weight complexes D-dimer therefore represent a prothrombotic state and testing is often requested when assessing venous thromboembolism comprising deep vein thrombosis and pulmonary embolism, and disseminated intravascular coagulation (Lippi et al., 2006).

The utility of D-dimer measurements is problematic, whilst a negative result excludes the likelihood of venous thromboembolism (VTE) a positive D-dimer may not be helpful in assessing a DVT or PE due to the high instances of positive results in a variety of clinical settings such as pregnancy, inflammation, malignancy, trauma, postsurgical treatment, liver disease (decreased clearance), and heart disease. It is also frequently elevated in hospitalised patients (Chopra, Doddamreddy, Grewal, & Kumar, 2012).

As a marker for hypercoagulability D-dimer is linked with thrombotic events in patients with venous as well as arterial thrombosis. D-dimer has also been implicated as an inflammatory marker as local fibrin formation and lysis are common events during inflammation (G. Lowe & Rumley, 2014). However elevated circulating D-dimer levels are not presently a specific inflammatory response marker.

As an inflammatory marker a number of studies have been undertaken to provide elucidation of the relationship with D-dimer and CVD. A steady significant rise in D-dimer (p < 0.002) has been observed during disease progression of pre-DM
to CVD complications but was related to levels of GSH and antioxidative activity (Nwose, Richards, Jelinek, & Kerr, 2007). This provides further supportive evidence that D-dimer may be a potential risk predictor for cardiovascular events, however it does remain unclear whether it is simply a marker or actually plays a causal role in the pathophysiology in these adverse events (Kleinegris, ten Cate, & ten Cate-Hoek, 2013). Whilst there are comparisons with D-dimer and several conventional risk factors and markers of inflammation, a lack of strong correlation would support the consideration that D-dimer may be viewed as informative as other conventional risk factors (Di Castelnuovo et al., 2013). There have been a number of studies associating D-dimer with CVD along with conflicting reports of hypercoagulation and DM. Sommeijer et al reported some coagulation markers are elevated in DM however when treating DM patients with statins a reduction in selected inflammatory markers was demonstrated with no significant reduction in levels of D-dimer (Sommeijer et al., 2004). It is recognised that in DM altered haemostasis results in a general increase in pro-coagulant factors with a concomitant decrease in fibrinolytic activity (Alzahrani & Ajjan, 2010). Surgically ill hospitalised patients with hyperglycaemia have demonstrated increased D-dimer levels (Duncan, 2012; Lemkes et al., 2010). The more recent discovered thrombin-activatable fibrinolysis inhibitor (TAFI) appears to be involved in development of the vascular endothelial damage associated with T2DM is suggestive that there is hypo-fibrinolysis, as thrombomodulin-thrombin complex, formed on intact vascular endothelium, may activate TAFI suggesting a possible link between plasminogen activator inhibitor (PAI-I) and TAFI. If this is correct it would indicate a possible reduction in D-dimer levels in DM, particularly in light of the observation of increased
synthesis of PAI-I in hyperglycaemia (Y. Yano et al., 2003). Increased levels of
the obesity marker PAI-1 demonstrated in hypercoagulable and hypofibrinolytic
individuals with CVD may also indicate the important role PAI-1 has in limiting
fibrinolytic activity and the increased risk of arterothrombotic events, particularly
in obese and metabolic syndrome patients (Phelan & Kerins, 2014). In our
studies despite recognising atherosclerosis as a hypercoagulable state the link,
albeit complex, with oxidative stress and vascular events cannot be ignored. A
link with GSH and T2DM and the degree of atherogenesis has been demonstrated
(Nwose, Jelinek, Richards, Tinley, & Kerr, 2009), and may be of assistance when
included in a panel of markers for oxidative stress but as a stand-alone marker
the lack of sensitivity in depicting normal haemostasis or fibrinolysis in
hyperglycaemia induced PAI-I is limited (E. U. Nwose, R. S. Richards, P. G.
Kerr, R. Tinley, & H. F. Jelinek, 2008a). An inverse relationship between D-
dimer and GSH has been demonstrated in diabetes, however studies with ankle
brachial pressure index (ABPI), used to differentiate between arterial disease and
peripheral vascular disease, supports the diverse nature of interaction between
coagulation, oxidative stress and inflammation in atherosclerosis and peripheral
vascular disease when assessing traditional and emerging biomarkers such as D-
dimer, GSH and IL-6 (H. F. Jelinek et al., 2015).

Conclusion

The emergent global pandemic of T2DM maybe pinnacle to underlying
predispositions such as pre-DM and MS, both of which may be preventable if
intervention is timely. The purpose of this review was to provide further insight
into the processes which contribute to the development of T2DM and CVD. It is widely recognised that T2DM and CVD is considered a progressive disease and can be considered multifactorial. The pathophysiology and pathogenicity of T2DM and CVD disease progression, amongst other things, incorporates oxidative stress, inflammation, hypercoagulative and hypofibrinolytic conditions. If these emerging biomarkers can provide further input into predictability and diagnosis of the hyperglycaemic pathologies, savings to the burgeoning costs to health budgets, and as a corollary lifestyle benefits improvements will be beneficial and potentially preventable. The importance of exercise with demonstrated improvements in comorbidities associated with T2DM is widely recognised and was therefore included in this study. The impact of inflammatory markers, including novel inflammatory ratios, explored in a control group undergoing endurance testing (ET) and high intensity interval training (HIIT) indicated that HIIT and its known cardiometabolic may provide benefits to hyperglycaemic patients. Lifestyle induced sedentariness and increasing obesity is still problematic globally.

The studies conducted during the course of this thesis point towards the need to continue research into the role of oxidative stress, inflammation and coagulability in the hyperglycaemic state. By increasing our understanding of how oxidative stress and inflammation participate in the development of the hyperglycaemic state will afford better and more reliable information required for the prediction and risk management of T2DM and CVD progression.

The following chapters develop the research work conducted in this thesis with each chapter presenting a published research article, or research manuscript in publication, which build upon and develop the theme in the investigation of
emerging biomarkers of oxidative stress, inflammation and coagulability. Whilst each publication stands alone each chapter contributes to the developing narrative of how oxidative stress and inflammation, through the emerging biomarkers we have analysed, contribute to the development of T2DM, hyperglycaemia, metabolic syndrome and CVD.
Chapter 2

Paper 1: Cardiovascular risk assessment in prediabetes: A hypothesis


At the commencement of this thesis the question asked was how oxidative stress, inflammatory and coagulation biomarkers participate in or contribute to the development of type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD). My role as Chief Medical Scientist and Operations Manager of the South Pathology Service (south west region of NSW), included the responsibility for the approval and oversight of all research activities conducted by the laboratory. As a PhD student I undertook, in this publication, to frame the medical hypothesis with Dr Nwose, an employee of the pathology service and Dr Richards, a senior lecturer at Charles Sturt University. My contributions were in the initial collegiate construction, writing, reviewing the background to the hypothesis, the discussion, editing and feedback to the other authors prior to approval for submission. Mr. Cann, as a trainee scientist, assisted in compiling the references and aiding in the review of the paper.

In this publication we recognised that currently two models for assessment and management of CVD are in use in Australia. One is premised on the Framingham Heart Study, the other is the New Zealand Guideline Group (NZGG). Our hypothesis was to provide an alternative model of assessment of diabetic macrovascular complications which would discriminate between prediabetes and
undiagnosed diabetes. In essence this should improve identification of persons that would not qualify for interventional treatment against CVD according to common screening models. Preliminary studies were reported that glutathione (GSH) and D-dimer were significant predictors of diabetes. GSH and D-dimer correlations indicated a link between oxidative stress and coagulation/fibrinolysis activity in diabetes and atherothrombotic activity. The coagulation disturbance is a consequence of the oxidative stress and inflammatory process. This paper proposed that emerging biomarkers such as GSH, malondialdehyde (MDA), D-dimer and homocysteine (Hcy), be considered along with the traditional markers. As different biomarker parameters reflect differing biochemical pathways, it was also recommended that an expanded sample size for biomarker analysis be conducted and that a longitudinal study be subjected to a binomial regression analysis to ‘refit’ lipid modelling and generate an exclusive model for screening CVD risk in subclinical diabetes. The foundation of this thesis has subsequently been systematically analysed, compared and correlated with emerging biomarkers, the hyperglycaemic state and CVD investigated.

Paper 2 followed through on emerging biomarkers where we investigated the balance between glutathione (GSH), glutathione sulphide (GSSG) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) and how this was disturbed in patients with impaired fasting glucose.
Cardiovascular risk assessment in prediabetes: A hypothesis

E.U. Nwose a,*, R.S. Richards b, N.G. Cann c, E. Butkowski a

a South West Pathology Service, 590 Smeaton Street, Albany, NSW 2640, Australia
b School of Community Health, Charles Sturt University, PO Box 765, Albany, NSW 2640, Australia

SUMMARY

There are screening programs for future risk of cardiovascular disease (CVD) complications in diabetes, but not in subclinical diabetes. There is little or no risk and no differences between genders when a man or woman at age below 50 years presents blood pressure below 140/90 mmHg and total cholesterol/HDL less than 7.0. In the current screening programs, a hypothetical apparently non-diabetic and non-smoking person aged 49 years old, who present blood pressure 140/90 mmHg, fasting blood sugar 5.8 mmol/L and total cholesterol/HDL 6.5 has no risk of future CVD and does not require any intervention. However, by counting numbers, the person has two risk factors, hyperglycaemia and hyperlipidaemia. Furthermore, considering smoking as a factor and the propensity for hyperglycaemia-induced oxidative stress being a smoker-like effect of hyperglycaemia toxicity, the person actually has three risk factors, which qualifies the person for intervention. The issue is that a prediabetes sufferer is treated like a healthy person in the current screening programs. The problem here is that risk of CVD in prediabetes is inadequately assessed. We present a hypothesis that employs a combination of blood glucose level and an index of oxidative damage to improve CVD screening in prediabetes. We propose a longitudinal study to repeat the whole lipid modelling exercise in order to develop a separate model chart for the screening of future CVD in people with diagnosed or undiagnosed prediabetes. The proposal would also serve for people with undiagnosed diabetes.

Background to hypothesis

The death rate due to diabetes mellitus (DM) has continued to increase, while deaths attributable to cardiovascular disease (CVD) are declining. Disconcertingly, the diabetic microvascular complications still constitute a major burden. Subclinical diabetes refers to a state of impaired fasting glucose, impaired glucose tolerance or prediabetes. This has been recognized as a factor to consider in the effort for early intervention against DM and its CVD complications [1,2]. It is now well reported that persons with prediabetes are at increased risk of developing CVD, in addition to developing diabetes [3,4]. The risk is perhaps due to an ongoing, but unmanaged, hyperglycaemic toxicity.

Metabolic syndrome refers to a group or disorders that are associated with increased risk of CVD and DM [1]. Whether the indices of metabolic syndrome is a predictive tool for future occurrence of diabetes is still an issue for debate. However, thromboembolism has been reported to be a manifestation of the metabolic syndrome [5]. This draws attention to diabetic hypercoagulability and whether plasma D-dimer can be employed for assessment of subclinical atherothrombosis in diabetes management.

Essentially, a significant factor to be addressed is the degree of risk of macrovascular complications in prediabetes. It has been suggested that an aggressive diagnostic approach among people with asymptomatic DM associated with one or more risk factors for coronary artery disease (CAD) is imperative [6]. Hypothetically, one of the options of aggressive diagnostic approach could be the determination of the changes in concentration of erythrocyte markers for oxidative stress associated with CVD and DM [7].

Hyperglycaemia-induced oxidative stress is primary to development of hypertension in diabetes

Deficiencies of the glutathione enzymes that metabolize reduced glutathione (GSH), including the glutathione peroxidase, reductase and synthetase, constitute a cause of non-spherocytic haemolytic anaemia [8]. The common manifestation of these enzymes' deficiencies is a drop in antioxidant potential of the erythrocyte-GSH. The effect is a sequence of erythrocyte oxidative stress (EOS), membrane damage, and red cell destruction [9]. A further consequence of excessive or progressive red cell destruction is anaemia and reduced bloodO2 supply (hypoxia), which leads to ischaemia and subsequent vasculopathy (Fig. 1) [10]. There is evidence of a close relationship between lipid peroxidation of human erythrocytes and haemolytic anaemia [11].
Hypoglycaemia

↑ Erythrocyte antioxidant activities
↓ Erythrocyte GSH concentration
↓ Erythrocyte oxidative stress
↑ Blood viscosity
↓ Haemolysis & vascularopathy
↓ Hypoxia (including cardiac tissue)
↑ Cardiac Output
↓ Hypertension & subsequent complications

Fig. 1. Schematic flow of how hypertension and subsequent complications can occur due to hypoglycaemia-induced erythrocyte oxidative stress.

On or before the development of vasculopathy, hypoxia is associated with increased cardiac output [12]. All things being equal, prolonged increase in cardiac output manifests as hypertension. Indeed, oxidative stress (OS) and anaemia have been demonstrated to concomitantly affect hypertension [13], and increased risk of diabetic macrovascular disease [14]. Hence, there are schools of thought advocating the use of oxygen supplementation to alleviate OS effects [15], and the concept of reducing CVD complications in DM via management of anaemia [16].

What has not been given adequate attention is the cellular mechanism of EOS arising from hyperglycaemia-induced depletion of GSH, which compromises the free radical scavenging function of the erythrocyte [16], and induce macr...}

Erythrocyte GSH and plasma D-dimer are indices of oxidative damage associated with diabetic macrovascular progression

There is a spectrum of traditional cardiovascular screening factors, cholesterol profile, OS indices and Virchow’s triad that hold promise as predictors of future macrovascular disease [18]. However, different biomarkers reflect different biochemical processes or pathophysiological pathways [19]. Therefore, the problem remains: how can the identifiable changes in the biomarkers, which are associated with the progression of diabetic macrovascular complications, be useful for screening ‘subclinical macrovascular disease’ in an individual who has ‘subclinical diabetes’?

We have presented a preliminary study report that erythrocyte GSH and plasma D-dimer levels are significant predictors of diabetic progression, even after adjusting for confounding factors [20]. The classical prediabetes individual, compared to a healthy individual, has higher levels of plasma D-dimer and lower levels of erythrocyte GSH. It is known that loss of erythrocyte GSH can compromise the erythrocyte’s free radical scavenging function [16], which then leads to EOS and the attendant vasculopathy including atherothrombogenesis [21]. Therefore, it is likely that blood glucose level, erythrocyte GSH and plasma D-dimer constitute a panel of indices that will provide diagnostic value on the OS status and oxidative damage of patients.

Induction of EOS reduces erythrocyte membrane fluidity that in turn increases both red cell aggregation and blood viscosity [21]. It is known that blood flow factors including viscosity and red cell aggregation are predictors of arterial thrombosis [22]. This explains how OS accelerates arterial thrombosis [23]. Based on this knowledge, central to our hypothesis is the concept that hyperglycaemia-induced depletion of erythrocyte GSH can cause a sequence of EOS (indicated by low levels of GSH and/or increased levels of malondialdehyde as a product of lipid peroxidation), impaired erythrocyte membrane fluidity and increased blood viscosity that enhances atherothrombogenesis and subsequent fibro-myolysis that is indicated by plasma D-dimer level.

We have investigated whether plasma D-dimer levels, an index of thrombotic activities, correlate with erythrocyte GSH as an index of EOS associated with diabetes [21]. The observed correlation reflects the level of association between (i) EOS (indicated by erythrocyte GSH) and (ii) the rate of coagulation/fibrinolysis activities (indicated by D-dimer levels). Since D-dimer and GSH are indices of atherothrombogenic activities and OS, respectively, and given the non-correlation in the control group, it is conceivable that the observation reflects the correlation between atherothrombogenesis and OS, which are both associated with diabetes [25].

Central to our hypothesis, adjunct to hyperglycaemia-induced depletion of erythrocyte GSH being primary to macrovascular complications of DM (Fig. 1), is that EOS is one of the underlying mechanisms of hypercoagulation in diabetes, which leads to macrovascular complications. Therefore, assessment of the usefulness of plasma D-dimer as a screening tool may serve to prove the validity of this variable after considering other confounding factors.

The problem: current cardiovascular disease screening programs do not provide for the subclinical or undiagnosed diabetes

Currently, two models are in use for the assessment and management of cardiovascular risk in diabetes. One is the flowchart based on the Framingham Heart Study [26]. The other, which is used in Australia and New Zealand, is the New Zealand Guidelines Group (NZGG) model (Fig. 2) [27]. Both models use diagnosis of diabetes, gender and smoking status as categorical variables; age, blood pressure readings and TC/HDL ratio as continuous variables.

It is noteworthy that the person with undiagnosed prediabetes is suffering hyperglycaemic toxicity similar to that which the diabetic patients and smokers suffer [28]. Since the Framingham flowchart and the NZGG models have diabetes and smoking status as dichotomous (‘YES’ or ‘NO’) variables, the prediabetic individual is, on the basis of ‘NO’ answer, categorized as a healthy non-diabetic and non-smoking.

There is little or no risk as well as no differences between genders for non-diabetics at age >50 years, blood pressure <140/90 mmHg and total cholesterol/HDL (TC/HDL) <2.7. Consider an instance where a man or woman has no diagnosis of diabetes, non-smoker, 42 years old, fasting blood sugar (FBS) level is 5.9 mmol/l, blood pressure 120/80 mmHg and TC/HDL 6.5. The 5-year CVD risk = <2.5, which means no risk and no need for intervention. Yet, the man or woman has FBS 5.9 mmol/l and TC/HDL 6.5. This is a concurrence of hyperglycaemia and hyperlipidaemia, which amounts to number of CVD risk factors = 2 being present. Furthermore, the man or woman may have antioxidant depletion due to...
the hyperglycaemia [29]. That is, a non-smoker having oxidative stress as in smokers [29]. Thus, there may be number of CVD risk factors equal to three being present, which makes the man or woman qualify for intervention.

This is evidence that individuals with prediabetes are not accommodated in the current screening programs. It implies that when health care providers use the either of the models to determine the risk of macrovascular complications in individuals with either prediabetes or undiagnosed diabetes, they are likely to be arriving at a false low risk. Therefore, there is need for a model that allows for assessment of macrovascular complications in prediabetes.

The hypothesis: alternative model of assessment of diabetic macrovascular complications that will discriminate prediabetes and undiagnosed diabetes

It has been investigated whether the probability of subclinical diabetic macrovascular complications occurring in prediabetes can be better related to a separate logistic model using the combination of conventional risk factors in the current screening models [20]. The binomial logistic regression study used a group of individuals with high blood glucose level (prediabetic state) as baseline, and another group of individuals with clinical diagnosis of both DM and CVD co-morbidity as the endpoint. It was observed that using the current conventional factors (i.e. DM and smoking as dichotomous variables, with blood pressure, TC/HDL ratio and age as continuous variables), a logistic equation could not be generated due to DM as an explanatory variable being an exact statistical surrogate for the endpoint. Therefore, a logical formula to determine probability of future CVD ‘outcome’ could not be generated [20]. This problem does not arise in the current screening programs that are primarily focused on diabetes individuals, because there is no surrogacy where there is DM at both the baseline and the endpoint.

When the logistic regression analysis was modified to omit DM factor and FBS level was substituted as a continuous variable, the modification successfully gave a logistic equation. Furthermore, when erythrocyte GSH levels was substituted for smoking variable, FBS and GSH were among the factors that entered into the logistic equation [20]. The report provides a rationale for an alternative risk model that includes FBS and erythrocyte GSH to enable adequate and better risk indications of subclinical diabetic macrovascular complications for individuals with prediabetes and/or undiagnosed diabetes.

Indeed, GSH had previously been suggested as an independent predictor of vascular disease [19]. The use of two or more conventional risk factors to identify persons with subclinical coronary disease has not been helpful, except in a more severe CAD [6,18]. We hypothesize that a model that substitutes FBS as a continuous variable for DM factor, and uses erythrocyte GSH level on the non-smoking individuals would provide a better CVD screening tool for individuals with prediabetes or undiagnosed DM.

Discussions

Potential implications

The objective is to establish and provide alternative and improved model to screen subclinical CVD complications in prediabetes and undiagnosed DM. The primary implication is to improve identification of persons that do not qualify for inter-
vention against CVD according to the current screening models, but who indeed have albeit emerging laboratory indications consistent with subclinical diabetic macrovascular complications. Another important goal is establishment of a diagnostic tool for assessment and management of oxidative damage in diabetic pathogenesis. The latter would provide evidence base to improve interventions with antioxidant and antioxidant therapies.

The need to identify and manage factors that are causally related to CVD has been imperative [30]. Therefore, given the reported likelihood of correlation between erythrocyte GSH and plasma D-<wbr/>dimer in diabetes, our proposal would generate evidence base to treat subclinical atherosclerosis in undiagnosed diabetes, as well intervene against CVD complications that might occur at the prediabetic stage.

Further more, it is known that a screening program developed with a particular population may not necessarily work well for another population. For instance, it has been reported that risk scores for type 2 diabetes developed with Caucasian populations varied widely, with sensitivity, specificity, and percentage in non-Caucasian populations [31]. Therefore, another potential implication is development of a screening program that is specific for the population where the study shall be performed.

Proposed tests — agenda for correction

Our hypothesis is strongly hinged on the fact that FBS and associated oxidative damage underlying diabetic macrovascular pathogenesis, are observable in the prediabetic state, and therefore should be considered as risk factors. FBS is the index of hyperglycemia toxicity in subclinical diabetes, instead of [yes] or [no] diabetes. Diagnostic indices of hyperglycemia-induced oxidative damage include erythrocyte GSH, malondialdehyde, plasma D-dimer and homocysteine. Considering the validity of conventional risk factors and the emerging laboratory factors such as erythrocyte GSH and malondialdehyde, as well as plasma D-dimer and homocysteine, there is now the spectrum of clinical signs and personal details, cholesterol profile, oxidative stress indices and Virchow’s triad indices. However, different parameters reflect different biochemical processes; not necessarily the same pathophysiological pathways. Hence we need to logically narrow down the population to a set of markers. We suggest agenda for correction to include:

1. Run larger sample-size studies to establish (i) prevalence of abnormal or positive D-dimer in prediabetes; (ii) correlation of D-dimer levels with red cell GSH levels in diabetes; and (iii) oxidative damage panel as a clinical diagnostic tool, including to standardize and validate methods for OS markers for use in clinical diagnostic use.

2. Run a longitudinal study with biomarker logistic regression analysis in order to re-fit the lipid modelling exercise, and generate an exclusive model for the screening of CVD risk in subclinical diabetes. In this proposed, we strongly recommend using (i) FBS as a continuous variable; and (ii) categorized oxidative stress panel (OSP) index, such as GSH and/or plasma D-dimer alongside with ‘smoking’ factor.

Conclusion

A separate model incorporating fasting blood sugar level and OSP index may improve the prediction of future diabetic macrovascular complications in prediabetic and undiagnosed DM individuals. This hypothesis is important for improved early intervention against the rising diabetes death toll.

References


Chapter 3

Paper 2: Glutathione:Glutathione sulfide redox imbalance in early impaired fasting glucose


In this publication my principal role as a co-author was collaborative and management of the analyses conducted, literature searches, commentary and input on the development of the paper and agreement of the final edition. To follow on from our medical hypothesis postulate in paper 1. As the emphasis of the thesis was to review the impact that oxidative stress (OS), inflammation and coagulability has had on the development of T2DM and CVD, this paper examined the balance between glutathione (GSH) and glutathione sulfide (GSSG) and how this was disturbed in impaired fasting glucose (IFG). 8OHDG another marker of OS was also included. OS is implicated in many clinical and progressive disease states and amongst these is the link metabolic syndrome (MS) may have with disorders due to the presence of hyperglycaemia. A measure of oxidative stress is in monitoring GSH and its oxidised form GSSG and calculating their ratio. With increased levels of blood glucose free radicals increase dramatically, GSH is protective and scavenges reactive species decreasing levels during states of oxidative stress resulting in GSSG. 8OHDG is also a sensitive marker of oxidative stress, but generally requires the presence of increased triglycerides, however there does remain some controversy with its association with lipids. The outcome of this study recognised that whilst markers such as GSH:GSSG and 8OHDG are linked to oxidative stress it may not be associated with disease progression;
whereas hyperglycaemia is consistent with oxidative stress. IFG is the preclinical stage of T2DM and is a useful target for early interventional therapies to prevent further disease development. A very important finding of this study was the significant decrease in the GSH:GSSG ratio ($p = 0.04$) in the IFG compared to the control group. 8OHdG levels are not so definitive and is controversial due to positive and negative associations with cholesterol. In this study we did not observe any significant difference. Further exploration on the interaction of glucose levels, oxidative stress, inflammation and pathological changes such as those that occur in T2DM and CVD therefore requires further investigation. In paper 3 we further explored OS and inflammatory marker correlations in T2DM and the Framingham CVD risk.
Glutathione: Glutathione Sulfide Redox Imbalance in Early Impaired Fasting Glucose

Herbert F. Jelinek, Hayder A. Al-Aubaidy, Laura Maschirow, Sarah Meidinger, Dina A. Jamil, and Eugene Butkowski

1 Australian School of Advanced Medicine, Macquarie University, Sydney, Australia.
2 Centre for Research in Complex Systems and the School of Community Health, Charles Sturt University, NSW, Australia.
3 Department of Biomedical Engineering, Khalifa University, Abu Dhabi, United Arab Emirates.
4 School of Medicine, University of Tasmania, Hobart, Australia.
5 Institute of Nutritional Sciences, University of Potsdam, Germany.
6 Department of Cellular Biology, University of Hamburg, Germany.

Authors’ contributions

This work was carried out in collaboration between all authors. Authors HFJ and HAAA designed the study, wrote the protocol. Authors LM and SM wrote the first draft of the manuscript. Authors EB and DAJ managed the literature searches and analyses of the study. All authors agreed on the final edition of the paper.

ABSTRACT

Aim: The current study aims to examine the balance between glutathione and glutathione sulfide and how this was disturbed in patients with impaired fasting glucose (IFG) level. The study also included 8-hydroxy-2'-deoxyguanosine to provide a more comprehensive picture of the overall redox state.

Methodology: A cross-sectional analysis of ninety medication free participants without reported history of cardiovascular disease and/or diabetes mellitus was undertaken with data collected from the Diabetes Complications Research Initiative database at Charles Sturt University. Fasting blood glucose, HbA1c and cholesterol as standard markers for diabetes mellitus and associated complications were measured in addition to the emerging biomarkers glutathione (GSH), glutathione disulfide (GSSG), and urinary 8-hydroxy-2'-deoxyguanosine (8OHdG).

*Corresponding author: E-mail: halaubaidy@yahoo.com;
**Results:** The IFG group had a mean blood glucose level above 6.1 mmol/L being significantly higher compared to control ($P<0.001$). Traditional clinical markers were all within the normal range for both groups. However, the GSH/GSSG ratio (8.53±5.4 vs 6.62±2.2, $P=0.04$) was significantly lower in the IFG group. GSH and 8OHdG, being markers for oxidative stress, were not significantly different between the two groups.

**Conclusion:** The free radical related changes in metabolic redox pathways are linked to oxidative stress and related pathologies but may not be associated with disease progression, providing an explanation why conflicting results are presented in the literature concerning any individual biomarkers and risk of diabetes. Our study included individuals with no medication use and mild hyperglycemia (impaired fasting glucose) and indicates a pro-oxidant response to mild-moderate hyperglycemia with a moderate rise in oxidative DNA damage.

**Keywords:** Impaired fasting glucose; oxidative stress; antioxidants; glutathione; GSH/GSSG ratio.

**1. INTRODUCTION**

Oxidative stress is implicated in diabetes mellitus, obesity, atherosclerosis, coronary artery disease, heart failure, and renal disease [1]. Metabolic syndrome may form a common link between these disorders due to hyperglycemia induced oxidative stress mechanisms. Glutathione is one of the main cellular antioxidants which has been found to increase in prediabetes and diabetes mellitus [2,3]. However the diversity of redox associated changes in redox signaling pathways associated with diabetes progression requires the identification of suitable reaction products that correlate with the pathophysiological processes associated with oxidative stress from normoglycemia to chronic hyperglycemia [4]. The oxidative stress can generate free radicals which are able to cause damage to the DNA, proteins and lipids [2–4]. These radicals react with guanine bases in DNA to form 8-hydroxy-2'-deoxyguanosine (8OHdG) [5]. Previous studies have shown that 8OHdG is associated with increased risks of diabetes mellitus [6;7] and atherosclerosis [8]. In our previuus studies, we observed serum 8OHdG was significantly elevated among the impaired fasting glucose (IFG) group compared to the control group [9].

**2. MATERIALS AND METHODS**

Data for this study was obtained from patients attending the diabetes complications clinic at Charles Sturt University, Australia. Participants were recruited via public media announcements. 428 participants (female: male, 247:181) were screened and after excluding those with diabetes mellitus, cardiovascular (CVD) or renal disease as well as those taking any medication, data from 62 control and 28 impaired fasting glucose participants were analyzed. Impaired fasting glucose was set at 5.6 mmol/L in accordance with the American Diabetes Association [10]. Anthropometric data was obtained (Table 1) in addition to blood glucose levels (BGL), and cholesterol profile. Differences in the level of biochemical markers of oxidative stress (erythrocyte reduced glutathione (GSH)/glutathione disulfide (GSSG)), endothelial dysfunction (urinary 8OHdG) and lipid were determined for IFG and control subjects.

After an overnight fast, whole blood specimens were collected into heparin and EDTA tubes for analysis. Plasma was separated within 1 hour by centrifugation at 1000xg for 10 min.
Plasma from heparin-containing tubes was immediately used for lipid analysis. Plasma from EDTA-containing tubes was kept at -80°C for serum 8OHdG and GSH analysis. Fasting plasma total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) were measured by standard techniques. TC and TG were determined with a commercial enzymatic kit. HDL-C was determined by immunoinhibition assay. Low-density lipoprotein cholesterol (LDL-C) was calculated according to the Friedewald formula [11]. Serum 8OHdG was measured using an EIA Kit, Cayman Chemical, MI, USA [12]. The test utilizes an anti-mouse IgG-coated plate and a tracer consisting of an 8OHdG-enzyme conjugate which detects all three oxidized guanine species; 8-hydroxy-2'-deoxyguanosine from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA. This format has the advantage of providing low variability and increased sensitivity compared with assays that utilize an antigen coated plate and only detect 8OHdG. Fresh blood was kept on ice for not more than 1 hour to measure GSH. The level of erythrocyte GSH was determined using the 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) reaction [13]. The GSH: GSSG ratio was determined using the formula (total GSH-2GSSG)/GSSG.

3. RESULTS

Participants were divided into two groups: (i) a control group with normal blood glucose levels and (ii) an IFG group with impaired fasting blood glucose according to the recommendations of the American Diabetic Association [4]. (Table 1) shows the main demographic characteristics of the two groups. The control and impaired fasting glucose subjects were comparable for the body mass index, and for systolic and diastolic blood pressure but the IFG group was significantly older. No participants were taking either antiarrhythmic, antihypertensive medication or statins.

| Table 1. Anthropometric results of the participants involved in this study |
|---------------------------|----------------|----------------|
|                          | Control (n=62) | IFG (n=28)     | P value   |
| Age (years)              | 62±11.5*      | 66.4±11        | ns**      |
| BMI (kg/m²)              | 26.4±       | 25.8±4         | ns        |
| SBP (mmHg)               | 120±14       | 124±13         | ns        |
| DBP (mmHg)               | 74±7         | 74±7           | ns        |

*means.d.; **nonsignificant; ^BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure

<table>
<thead>
<tr>
<th>Table 2. Biomarker results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>BGL (mmol/L)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
</tr>
<tr>
<td>GSH (mg/100ml)</td>
</tr>
<tr>
<td>GSSG (mg/100ml)</td>
</tr>
<tr>
<td>GSH:GSSG</td>
</tr>
<tr>
<td>8OHdG (ng/ml)</td>
</tr>
</tbody>
</table>

*means.d.; **non significant; ^BGL = blood glucose level, HbA1c = glycated hemoglobin, TC = total cholesterol, HDL-C = high-density lipoprotein-cholesterol, LDL-C = low density lipoprotein-cholesterol, GSH = glutathione, GSSG = glutathione disulfide, 8OHdG = 8-hydroxy-2'-deoxyguanosine
(Table 2 above) shows the biomarker results for the study. There were no significant differences in blood lipid levels between the two groups (p>0.05). Reduced GSH and glutathione disulfide were not significantly different between the two groups, nor was 8OHdG. The GSH/GSSG ratio was significantly (p<0.05) decreased in the IFG group.

4. DISCUSSION

IFG is the preclinical stage of diabetes mellitus and is a useful target for early intervention therapies to prevent the development of this wide-spread disease and its associated complications. We focused on investigating biomarkers associated with moderate increases in BGL as observed for IFG in individuals with no CVD, hypertension or kidney dysfunction and free of medication. Based on the definition of IFG, we expected to find adverse effects of mild hyperglycaemia in this group. However the HbA1c, blood lipid levels and blood pressure did not differ between the groups.

Increased blood sugar levels are associated with oxidative stress leading to a myriad of free radical formation and antioxidant response [14-16]. Free radicals, non radical oxidants and non radical-thiol-reactive chemicals all play a role in the multiple metabolic pathways associated with oxidative stress and diabetes mellitus [17].

A very important finding of this study is the significant decrease in GSH/GSSG ratio in the IFG group compared to control. The significant difference in GSH/GSSG between the two groups may be indicating an early thiol related oxidative stress response and impaired redox status with erythrocyte GSH levels slightly up but close to the control group. This is consistent with our earlier findings and those reported in the literature, which showed that changes in the antioxidant status, especially the erythrocyte glutathione system, characterize the initial phase of oxidative stress in diabetes mellitus progression and commences prior to the establishment of the disease when BGL are above 7mmol/L [18-20]. Reduced glutathione is an ubiquitous antioxidant, which has been shown to be lower in IGT subjects with similar levels to that reported in frank diabetes mellitus [20,21]. Glutathione protects against peroxides and toxic aldehydes by undergoing oxidation to GSSG. However lower GSH and higher GSSG levels are not only associated with disease processes but also with aging, where the GSH pool is more oxidized [22,23]. From our data it can be seen that the GSH pool size and reduced GSH remained relatively constant between controls and IFG participants. This suggests that age-related changes do not play a major role in the current cohort and the glutathione redox system is reacting to the increases in blood glucose levels associated with IFG, keeping oxidative stress to a minimum.

Previous work of ours has shown that although a decrease in reduced GSH is seen in IGT, the change was not significant [9] and may be associated with lipid peroxidation and endothelial damage in the vascular walls, which increases homocysteine and therefore reduces cysteine availability to GSH. Dincer et al. reported that oxidative stress inhibits the activity of glutathione reductase, the enzyme that regenerates GSH from GSSG [24], an effect, which could have contributed to the increased GSSG levels in our study. In the current study we demonstrate a significant reduction in GSH:GSSG redox balance indicating oxidative stress. GSH:GSSG may be a robust early marker of oxidative stress associated with impaired fasting glucose in the presence of a normal cholesterol profile and has been associated with type 2 diabetes mellitus.

We have also previously shown that 8OHdG is increased significantly in IFG but requires an increase in triglycerides. However this association between cholesterol and 8OHdG is
controversial with some authors showing positive and others negative associations to cholesterol levels [25-28]. In our work a drop in HDL or increase in LDL does not seem to affect 8OHdG when BGL is below 7mmol/L and HbA1c below 6.4% [29]. The current study still indicates this increase in 8OHdG, although not to a significant extent. These findings highlight the complex cellular interactions associated with diabetes mellitus and diabetes progression.

The pathophysiology of diabetes mellitus progression and associated changes in multiple biomarkers do not constitute a separate linear model for each biomarker such as HbA1c, lipids, oxidative stress and inflammatory markers but are a part of a complex interactive model, which changes depending on additional factors such as age and comorbidity [30]. Our model of mild IFG, within this framework of redox balance, suggests that redox balance can be impaired due to minor increases in blood glucose levels, which are not enough to decrease erythrocyte GSH or increase GSSG levels but do affect the GSH/GSSG balance. Changes in the GSH/GSSG redox state have been proposed to occur also in the absence of oxidative stress and depending on the level gluconeogenesis [31-35].

5. CONCLUSION

The free radical related changes in metabolic redox pathways are linked to oxidative stress and related pathologies but may not be associated with disease progression, providing an explanation why conflicting results are presented in the literature concerning any individual biomarkers and risk of diabetes mellitus. Our study included individuals with no medication use and mild hyperglycemia (IFG) and indicates a pro-oxidant response to mild-moderate hyperglycemia with a moderate rise in oxidative DNA damage.

CONSENT

All authors declare that written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Human Research Ethics Committee, Charles Sturt university and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


© 2014 Jelinek et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?id=545&id=26&aid=4865
Chapter 4

Paper 3: Diabetes, oxidative stress and cardiovascular risk


In this paper, I had the privilege of training and supervising Ms. L. Brix, a post graduate science intern, from Germany. I supervised and provided her laboratory training for the analysis of the biomarkers. The construction and preparation of this publication occurred with her assistance. Ms. Brix also assisted in the data collation and reduction. Dr’s Al-Aubaidy, Jelinek and Professor Kiat provided advice, direction and assistance in the introduction and discussion and reviewing of the article prior to submission for publication.

Building upon our previous papers this publication expanded upon correlations between oxidative stress (OS), inflammatory markers and applied to T2DM and the Framingham CVD risk score. Increased risk of complications are recognised in the prediabetic state, however they may go undiagnosed until T2DM is clinically evident. Prediabetes is defined as impaired fasting glucose (IFG). OS is associated with the hyperglycaemic state and is explored in this study in conjunction with CVD risk. The development of atherosclerosis is a result of injury, inflammation and an immune response indicating multiple independent pathways as contributing. OS and inflammation was explored in the 2 groups (Diabetic and CVD risk), along with other traditional markers and anthropometric data. Significant differences were observed in a number of traditional and emerging markers.
Of primary interest was GSH, GSH:GSSG and interleukin 6 (IL-6) in the diabetic and the CVD risk groups. GSH:GSSG was significant between control and prediabetic group (p <0.05) and IL-6 was significant in both prediabetic and T2DM group (p <0.05). GSH was significant (p <0.05) in the moderate and high CVD risk and IL-6 in the moderate risk (p<0.05) group. This study further highlighted the importance of OS and inflammatory markers as indicators of T2DM progression. A novel finding was the significant results of GSH:GSSG and IL-6 in the T2DM group and that GSH was significant with respect to CVD status providing further substance to the importance emerging biomarkers continue to contribute to our understanding. In paper 4 biomarkers were investigated in preclinical increases in blood glucose with a cohort which were not discriminated on the basis of medication.
Full Length Research Paper

Diabetes, oxidative stress and cardiovascular risk

Eugene G. Butkowski¹, Lea M. Brix¹,², Hosen Kiat³, Hayder A. Al-Aubaidy⁴, Herbert F. Jelinek¹,³,⁴

¹School of Community Health, Faculty of Science, Charles Sturt University, Albury, NSW, Australia,
²Department of Biology, Ludwig-Maximilians-University, Munich, Germany
³School of Medicine, University of New South Wales and Faculty of Medicine and Health Sciences, Macquarie University, Sydney, Australia
⁴School of Medicine, University of Tasmania, Hobart, Australia.

* Corresponding author email: HJelinek@csu.edu.au
Accepted 21 January, 2016

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 (Schrövers 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-Aubaiady 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-Aubaiady and Jelinek, 2011; Ballatori et al., 2000). 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 (Sannai 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 pathogenesis 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-Aubaiady 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 (Abram 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 ≥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 ethylenediamine 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-2-nitrobenzoic 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 EnzymeLinked Immunosorbent Assay (ELISA) kit (Elsaskit.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 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

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Prediabetes</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.02 ± 9.53</td>
<td>66.80 ± 10.98</td>
<td>72.26 ± 11.18</td>
</tr>
<tr>
<td>Sex (Female/Male)</td>
<td>21/15 (n=66)</td>
<td>8/6 (n=20)</td>
<td>3/6 (n=9)</td>
</tr>
<tr>
<td>BGL (mmol/L)</td>
<td>5.12 ± 0.38</td>
<td>6.09 ± 0.37</td>
<td>7.74 ± 2.59</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.62 ± 0.44</td>
<td>5.71 ± 0.39</td>
<td>7.00 ± 0.10</td>
</tr>
<tr>
<td>Smoking (% yes)</td>
<td>0</td>
<td>13.33 %</td>
<td>0</td>
</tr>
<tr>
<td>Alcohol (% yes)</td>
<td>2.78 %</td>
<td>6.67 %</td>
<td>33.33 %</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>119 ± 12</td>
<td>122 ± 13</td>
<td>129 ± 11</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>73 ± 7</td>
<td>74 ± 7</td>
<td>74 ± 7</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73m²)</td>
<td>92.45 ± 7.03</td>
<td>90.78 ± 22.42</td>
<td>90.95 ± 18.69</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.37 ± 1.92</td>
<td>5.08 ± 1.57</td>
<td>3.76 ± 0.67</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.00 ± 0.67</td>
<td>1.02 ± 0.62</td>
<td>1.20 ± 0.65</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.89 ± 0.36</td>
<td>1.86 ± 0.45</td>
<td>1.75 ± 0.76</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.39 ± 0.82</td>
<td>3.19 ± 0.82</td>
<td>2.02 ± 0.87</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>23.43 ± 16.34</td>
<td>20.28 ± 14.47</td>
<td>41.79 ± 40.07</td>
</tr>
<tr>
<td>GSH (μmol/L)</td>
<td>1632.46 ± 613.87</td>
<td>1515.85 ± 592.83</td>
<td>1307.51 ± 606.81</td>
</tr>
<tr>
<td>GSSG (μmol/L)</td>
<td>357.62 ± 244.86</td>
<td>416.16 ± 177.32</td>
<td>375.08 ± 188.99</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>7.80 ± 6.09</td>
<td>4.20 ± 2.08</td>
<td>4.04 ± 2.44</td>
</tr>
</tbody>
</table>

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 < 0.001).
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 = cardiovascular disease; 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 Jelínek, 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 (Jelínek et al., 2014; Kadiska et al., 2001; Mascherov et al., 2015). Previously Al-Aubaidy and Jelínek (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 Jelínek, 2011; Jelínek et al., 2014; Nwose et al., 2006; Nwose, Jelínek et al., 2007; Nwose et al., 2008).

The increased erythrocyte generated ROS observed with diabetes progression and CVD risk enhances
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 (Dandoneta et al., 2004). The results from Dandoneta’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 glutaryl 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 (Correti et al., 2006).

Table 2. CVD risk groups

<table>
<thead>
<tr>
<th>Low risk</th>
<th>Moderate risk</th>
<th>High risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.2 ± 9.66</td>
<td>73.0 ± 7.0 a</td>
</tr>
<tr>
<td>BGL (mmol/L)</td>
<td>5.40 ± 0.62</td>
<td>6.29 ± 1.71 b</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.58 ± 0.42</td>
<td>6.03 ± 0.86 b</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>89.13 ± 10.58</td>
<td>94.44 ± 12.59</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.57 ± 4.83</td>
<td>25.11 ± 1.76</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>118 ± 11</td>
<td>133 ± 12 b</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>73 ± 6</td>
<td>74 ± 6</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73m²)</td>
<td>94.09 ± 16.82</td>
<td>84.21 ± 26.08</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.62 ± 0.82</td>
<td>4.98 ± 1.00 b</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.10 ± 0.60</td>
<td>1.20 ± 0.43</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.75 ± 0.53</td>
<td>1.72 ± 0.59</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.38 ±0.78</td>
<td>2.70 ± 0.80 b</td>
</tr>
<tr>
<td>GSH (µmol/L)</td>
<td>1516.16 ± 622.31</td>
<td>1930.41 ± 595.94 b</td>
</tr>
<tr>
<td>GSSG (µmol/L)</td>
<td>322.15 ± 177.00</td>
<td>405.09 ± 143.58</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>7.14 ±5.86</td>
<td>5.40 ± 2.77</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>22.46 ± 14.40</td>
<td>38.07 ± 37.93 b</td>
</tr>
</tbody>
</table>

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).
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; Charman 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.

ACKNOWLEDGEMENTS

Lea Marie Brixi was in receipt of a DAAD (Deutscher Akademischer Austauschdienst) scholarship. Technical assistance from Bev de Jong and statistical advice from Simon McDonald (Statistical Analysis Network) is gratefully acknowledged.

REFERENCES


Published by Basic Research Journal of Medicine and Clinical Science.
Chapter 5

Paper 4: Hyperglycaemia, oxidative stress and inflammatory markers


My role in the development of this paper was in the design, construction and write up with advice, assistance in write-up of the discussion and feedback from Dr Jelinek. Additional statistical parametric and nonparametric analysis was performed by the Charles Sturt University spatial analysis unit.

This paper examined traditional and emerging biomarkers and their association with increasing blood glucose concentrations. The study group was divided into 1st and 4th, 5th quintiles based upon fasting blood glucose (FBG) levels. Anthropometric data waist circumference (WC) and body mass index (BMI) were significant between groups (p<0.001) as was general biomarkers – BGL, HbA1c, Triglyceride, High density lipoprotein (HDL), Total Cholesterol/HDL and atherogenic index (p<0.001). The emerging markers compared the 1st and 4th, 5th quintile with significant results observed for GSH (p <0.001), GSH:GSSG and 8OHdG (p <0.05). Inflammatory markers were also expressed as ratio and the 1st and 4th, 5th quintiles compared with significance for IL-1β/IL-10, IL-6/IL-1β and CRP/IL-6 (p <0.05).

The constellation of OS and inflammation emerging from and participating with the pro-inflammatory hyperglycaemic state is still not fully understood, however the progression to T2Dm and associated comorbidity still presents as a clinical dilemma. Our previous work has demonstrated OS and IL-1β disturbances in hyperglycaemia and further confirmed in this study,
additionally we have established in this study that results from a screening population have replicated the findings of well controlled groups. This will be explored further with a larger subject cohort. To our knowledge this is the first time ratio analysis of these biomarkers in hyperglycaemia has been explored. These novel findings will also be further investigated.

As this paper did not discriminate on the basis of medication our next publication (paper 5) investigated medication use with respect to metabolic syndrome.
Hyperglycaemia, oxidative stress and inflammatory markers

Butkowski, E.G.\textsuperscript{1}, and Jelinek, H.F.\textsuperscript{1,2}
\textsuperscript{1} School of Community Health, Charles Sturt University, Albury, Australia.
\textsuperscript{2} School of Medicine, University of New South Wales and Faculty of Medicine and Health Sciences, Macquarie University, Sydney, Australia

Address for Correspondence
Herbert F. Jelinek
School of Community Health
Charles Sturt University
Albury 2640
Australia
E: hjelinek@csu.edu.au

Abstract

Introduction: The increasing prevalence of diabetes and its association with a hyperglycaemic state implicates a state of oxidative stress and inflammation. Clarification of the role emerging biomarkers contribute to impaired glucose tolerance, metabolic syndrome and Type 2 Diabetes Mellitus requires further elucidation. This study reviews a number of traditional and emerging biomarkers associated with increasing blood glucose concentrations.

Results: Significant results were obtained in the traditional anthropometric data (waist circumference, body mass index and blood pressure) and biochemical markers - blood glucose, haemoglobin A1c and total cholesterol ($p < 0.001$). Emerging biomarkers were assayed and blood glucose 1\textsuperscript{st} and 4,5\textsuperscript{th} quintile groups statistically compared for significance with glutathione ($p < 0.001$), glutathione:glutathione disulfide ($p < 0.05$), 8-hydroxy-2’-deoxyguanosine ($p < 0.05$) and interleukin (IL) IL-1\textbeta ($p < 0.05$) IL-1\textbeta/IL-10, IL-6/IL-1\textbeta; CRP/IL-6 also provided significant results.

Conclusion: This study provided further evidence that emerging inflammatory and oxidative stress biomarkers may contribute to diagnostic information associated with preclinical increases in BGL as part of a
screening clinic. Further we have provided a unique study in the analysis of ratios of inflammatory biomarkers and any correlation with increased BGL.

**Keywords:** Type 2 Diabetes Mellitus, impaired fasting glucose, prediabetes, cardiovascular disease, oxidative stress, risk factors, body mass index, glutathione, glutathione disulfide, 8-hydroxy-2′-deoxyguanosine, interleukin 1β, interleukin-6, interleukin 10, monocyte chemoattractant protein 1, insulin like growth factor 1

**Introduction:**

Alterations to homeostatic disturbances in glucose metabolism and resultant hyperglycaemia are causative factors for Type 2 diabetes mellitus (T2DM). Chronic sustained hyperglycaemia also results in micro and macrovascular complications occurring through a number of mechanisms of which oxidative stress and inflammatory changes via the innate immune system have increased in interest for medical diagnostics (Brownlee, 2001; Collier, Dossett, May, & Diaz, 2008). Impaired fasting glucose (IFG) may lead to the development of T2DM and cardiovascular disease (CVD), associated with increased oxidative stress (Yan, Ramasamy, Naka, & Schmidt, 2003) along with the ensuing chronic subclinical inflammatory processes apparent with developing insulin resistance (S. M. Haffner, 2003). The challenge is to prevent complications associated with IFG of which approximately 30% will remain undiagnosed for a significant period of time (Gholap, Davies, Mostafa, & Khunti, 2013). Complications of T2DM continue to reduce the quality of life in the general community and places an increasing burden on the global health budget. The International Diabetes Federation have recognised the importance of the traditional biomarkers such as blood glucose (BGL), haemoglobin A1c (HbA1c) and lipid studies, recommendations for life style improvement and drug treatment regimens to combat T2DM and CVD (Federation, 2012). However emerging biomarkers and their relationship with the causation of T2DM and CVD are worthy of extensive investigation to aid in clarification of the role they play and their potential to provide information which may allow for early prediction and intervention.
**Hyperglycaemia and oxidative stress:**

Chronic hyperglycaemia is considered to be a major causative factor in the establishment of microvascular and macrovascular complications observed in T2DM. Reactive oxygen species, (ROS) as a result of hyperglycaemia, are known to damage nucleic acids, lipids and proteins with the degree or extent of damage related to the duration of the hyperglycaemia (Al-Aubaidy & Jelinek, 2010; Tatsch et al., 2012). The associated pathophysiological mechanisms which occur are suggestive of the excess generation of oxygen and nitrogen species and concomitant oxidative stress (Bandeira et al., 2013).

Oxidative stress (OS) is initiated by hyperglycaemia as a result of circulating intracellular and extracellular free radical levels (Al-Aubaidy & Jelinek, 2011a; Whiting, Kalansooriya, Holbrook, Haddad, & Jennings, 2008). Glutathione (GSH), is a responsive ubiquitous antioxidant and detoxifier of excessive free radicals and reactive oxygen species (Al-Aubaidy & Jelinek, 2011a; Ballatori et al., 2009). GSH is readily oxidised to glutathione disulfide (GSSG) making it the major physiological redox couple (G. Wu, Fang, Yang, Lupton, & Turner, 2004). Any oxidation of GSH and a consequential increase in GSSG enables the GSH:GSSG ratio calculation, which can be utilised as a useful marker in determining an antioxidant status (H. F. Jelinek et al., 2014). As decreased levels of GSH have been observed as a consequence of hyperglycaemia, primarily as a result of cysteine depletion and loss of cysteine assisted membrane transport mechanisms, cellular oxidative stress may increase (Bannai & Tateishi, 1986; Toroser & Sohal, 2007). Oxidative damage to DNA and RNA occurs due to nuclear free radical formation as a result of hydrogen peroxide oxidation. The interaction of the hydroxyl radical with the DNA nucleobase guanine forms the product C8-hydroxyguanine (8-OHGua) or the nucleoside 8-hydroxy-2’-deoxyguanosine (8OHDG) (Valavanidis et al., 2009). Increased levels of 8OHDG have previously been shown to be useful biomarkers in the assessment of atherosclerosis and T2DM (L. Maschirow et al., 2015). Oxidative stress and inflammation go hand in hand. The inflammatory response as a result of hyperglycaemia has a strong association with increased reactive oxygen species (ROS) (de Carvalho, Guedes, Goncalves, & de Cassia Goncalves, 2012; Y. Lin et al., 2005). There is therefore an imperative to observe levels of both oxidative
and inflammatory markers in the investigation of the hyperglycaemic state and the progression to T2DM and CVD.

**Hyperglycaemia and Inflammation:**

T2DM is characterised by hyperglycaemia primarily associated with insulin resistance but often obesity, dyslipidaemia, hypertension and accelerated atherosclerosis are clinical comorbidities (Black, 2003). T2DM has been further classified as a chronic inflammatory state with evidence of disparate concentrations of cytokines and acute phase reactants (APR’s) (Dallmeier et al., 2012). This inflammatory process and the association with T2DM is also contributory, but not necessarily exclusive to the progression to CVD (Schöttker et al., 2013). More traditional inflammatory markers such as C-reactive protein (CRP), are recognised and utilised as a general high sensitivity systemic marker of inflammatory processes (Salazar et al., 2014) and may be of some prognostic use in areas such as predicting coronary heart disease (Koenig et al., 1999). However CRP lacks specificity but in combination with other inflammatory and oxidative stress markers may improve risk prediction for T2DM and CVD as utilising current global risk assessment strategies and scores still remain sub-optimal (Herder et al., 2011). The inflammatory markers interleukins-1β, 6, 10 (IL-1β, IL-6, IL-10), monocyte chemo-attractant protein-1 (MCP-1) and insulin like growth factor-1 (IGF-1) were explored in this study. Chronic low-grade inflammatory responses with activation of the innate immune system have been associated with diabetes, metabolic syndrome (MS) and atherosclerosis (Esposito & Giugliano, 2004; Kristiansen & Mandrup-Poulsen, 2005; Plutzky, 2001). Association between inflammation, oxidative stress and BGL have not been comprehensively investigated (de Rekeneire et al., 2006; F. M. Schmidt et al., 2015; Spranger et al., 2003) and utilising these markers to classify participants attending a diabetes health screening clinic into those with possible preclinical CVD is by no means definitive. This study investigated the association of inflammatory and oxidative stress markers with normal BGL of <4.5mmol/L and those with increased BGL starting at the impaired fasting glucose cut-off >6.1mmol/L.
Methods:

Three hundred and nine participants were recruited from the Diabetic Health Screening Clinic (DiabHealth) at Charles Sturt University. The study was approved by the Charles Sturt University Human Ethics Committee (Protocol Number 2006-042) and complies with the standards of the Helsinki agreement for human research. All patients were informed of the aims of the research and any risk prior to consent. The control group consisted of volunteers with no evidence of T2DM, CVD, hypertension (HT). Anthropometric data was obtained (Table 1) in addition to blood and urine specimens. Specimens collected were analysed for blood glucose and lipids. Biomarkers for oxidative stress and inflammation were performed on all patient specimens. As this was a screening clinic clients were not discriminated and excluded based on medical and medication history. Body mass index (BMI) was measured using standardised beam weight scales. BMI is defined as weight in kilograms per height expressed as meters squared and is independent of gender and age. Waist circumference (WC) was measured using a standard measuring tape. Measurements were taken between the top of the hip bone and lowest rib. Blood pressure was measured using a sphygmomanometer with appropriate cuff size after a 5-minute rest. The average of two measurements 1 minute apart was used for systolic and diastolic blood pressure values.

Specimen collection and processing:

Patients fasted overnight prior to data and blood collection. Whole blood specimens were collected into plain, heparin and EDTA anticoagulated, 7 mL tubes. Serum and plasma was separated after a 10 minute centrifugation at 1000g. Glucose, total cholesterol, triglycerides, HDL cholesterol were performed at the local pathology laboratory, in accordance with Australian Laboratory Standards. All specimens were analysed immediately after collection. GSH, GSSG, 8OHDG and interleukins were determined on all subjects. Plasma/serum/washed red cell lysate analytes not tested immediately were stored at -80°C.
General biochemistry:

Plasma glucose, CRP, HbA1c, total cholesterol (TC), and triglycerides (TG) were determined by standard enzymatic kits and high-density lipoprotein (HDL) was determined using an immunoinhibition assay. Low-density lipoprotein cholesterol (LDL-C) was calculated according to the Friedewald formula. These investigations were carried out at Dorovitch laboratory, a national accredited private pathology laboratory.

Oxidative stress markers:

GSH and GSSG levels were measured from erythrocyte lysate by a Glutathione Assay Kit (Cayman Chemical, MI, USA). As the method incorporates glutathione reductase, total glutathione is measured. GSH reacts with Ellman’s reagent with the production of 5-thio-2-nitrobenzoic acid (TNB). Absorbance was measured at 405nm with the absorbance of TNB directly proportional to the GSH concentration in the sample. The GSH:GSSG ratio was determined using the formula (total GSH-2GSSG)/GSSG). Results were calculated using a four parameter logistic fit.

Plasma from 8OHdG was assayed with an enzyme immunosorbent assay (EIA) Kit (Cayman Chemical, MI, USA). The test procedure utilises an anti-mouse IgG-coated plate and a tracer consisting of an 8OHDG-enzyme conjugate which detects all three oxidized guanine species; 8OHDG from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA. This kit analysis has the advantage of providing low variability and increased sensitivity compared with assays that utilise an antigen coated plate which only detect 8OHdG.

Inflammatory markers:

IL-1β, IL-6, IL-10, MCP-1 and IGF-1 levels were determined using Enzyme Linked Immunosorant Assay (ELISA) kits (Elisakit.com, Adelaide Australia). The methods incorporated pre-coated IL, MCP & IGF capture antibody incubated with their appropriate anti-human (i.e. IL-1β, IL-6, IL-10, MCP-1, IGF-1) 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:**

Data analysis with descriptive data expressed as mean ± standard deviation (x ± SD), for demographic and anthropometric data and median ± IQR (interquartile range) and for nonparametric data analysed after input into Microsoft Excel (Office 2013). Parametric and nonparametric statistical analysis was performed with PAWS (Version 22, IBM Co) depending on whether data was normally distributed or not. Data was grouped by fasting blood glucose (FBG) <4.5mmol/l (1st quintile) vs FBG >6.1mmol/L (4th, 5th quintile) and compared, with results of p <0.05 considered significant.

**Results:**

The control group, who had no reported hypertension, CVD or diabetes as well as being medication free is shown as a comparison to the clinic groups (Table 1). Results were expressed as mean ± SD. Gender distribution was slightly higher for females (20M/27F) but not significant.
Table 1: Control group, mean and SD

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control group Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>20M/27F</td>
</tr>
<tr>
<td>Age</td>
<td>61.2 ± 9.9</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>91.8 ± 12.5</td>
</tr>
<tr>
<td>BMI</td>
<td>26.0 ± 4.6</td>
</tr>
<tr>
<td>SBP (mm/Hg)</td>
<td>125.5 ± 15.2</td>
</tr>
<tr>
<td>DBP (mm/Hg)</td>
<td>76.7 ± 7.4</td>
</tr>
<tr>
<td>BGL (mmol/L)</td>
<td>5.2 ± 0.9</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>5.3 ± 0.8</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.22 ± 0.8</td>
</tr>
<tr>
<td>TC/HDL ratio</td>
<td>3.4 ± 1.1</td>
</tr>
</tbody>
</table>


Table 2 depicts the study group as divided into 1\textsuperscript{st} and 4\textsuperscript{th}, 5\textsuperscript{th} quintiles based upon an FBG level. Anthropometric data WC and BMI were significant between groups ($p < 0.001$) as was the general biomarkers BGL, HbA1c, TG, HDL, TC/HDL ratio and AIP ($p < 0.001$).

Table 2: Anthropometric and General Biomarkers

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>BGL&lt;4.5mmol/L</th>
<th>BGL &gt;6.1mmol/L</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>19M/27F</td>
<td>49M/50F</td>
<td>ns</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>69.8 ± 12</td>
<td>69.3 ± 9</td>
<td>ns</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>91.8 ± 15.2</td>
<td>104.2 ± 14.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m\textsuperscript{2})</td>
<td>25.9 ± 5.4</td>
<td>29.9 ± 5.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP (mm/Hg)</td>
<td>130 ± 19</td>
<td>135 ± 19</td>
<td>ns</td>
</tr>
<tr>
<td>DBP (mm/Hg)</td>
<td>74 ± 10</td>
<td>78 ± 19</td>
<td>ns</td>
</tr>
<tr>
<td>AIP</td>
<td>-0.19 ± 0.2</td>
<td>0.08 ± 0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BGL (mmol/L)</td>
<td>4.5 ± 0.3</td>
<td>8.6 ± 3.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.6 ± 0.5</td>
<td>6.7 ± 1.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>5.0 ± 0.9</td>
<td>4.7 ± 1.3</td>
<td>ns</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.1 ± 0.5</td>
<td>1.8 ± 0.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.8 ± 0.5</td>
<td>1.4 ± 0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.7 ± 0.9</td>
<td>2.6 ± 1.1</td>
<td>ns</td>
</tr>
<tr>
<td>TC/HDL ratio</td>
<td>3.0 ± 0.9</td>
<td>3.8 ± 1.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Mean ± SD, Q1 represents glucose of <4.5mmol/L, Q4,5 represents glucose >6.1mmol/L, ns – not significant, WC – waist circumference, BMI – Body mass index, SBP – systolic blood pressure, DBP – diastolic blood pressure, AIP – atherogenic index, BGL – blood glucose level, HbA1c – Haemoglobin A1c, TC – total cholesterol, TG – triglyceride, HDL – high density lipoprotein, LDL – low density lipoprotein.
The comparison of oxidative stress and inflammatory markers (Table 3) compared the 1st and 4th–5th quintile. Significant results were observed for GSH \( (p<0.001) \), GSH:GSSG and 8OHDG and the inflammatory marker IL-1β \( (p<0.05) \).

**Table 3**  
Oxidative stress and inflammatory markers

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control Group*</th>
<th>Q1</th>
<th>Q 4,5</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (μmol/L)</td>
<td>1760.2 ± 729</td>
<td>2267.8 ± 673</td>
<td>1632.2 ± 612</td>
<td>(&lt;0.001)</td>
</tr>
<tr>
<td>GSSG (μmol/L)</td>
<td>286.4 ± 266</td>
<td>266.6 ± 110</td>
<td>352.4 ± 211</td>
<td>ns</td>
</tr>
<tr>
<td>GSH:GSSG ratio</td>
<td>5.8 ± 6</td>
<td>8.1 ± 4</td>
<td>5.1 ± 5</td>
<td>(&lt;0.05)</td>
</tr>
<tr>
<td>8OHDG (pg/mL)</td>
<td>511.9 ± 264</td>
<td>612.5 ± 429</td>
<td>865.2 ± 512</td>
<td>(&lt;0.05)</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>2.85 ± 5</td>
<td>2.48 ± 1.5</td>
<td>3.56 ± 8.5</td>
<td>(&lt;0.05)</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>19.19 ± 21.9</td>
<td>19.43 ± 24.2</td>
<td>15.72 ± 29.7</td>
<td>ns</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>19.95 ± 21.1</td>
<td>19.95 ± 115.8</td>
<td>18.51 ± 25.1</td>
<td>ns</td>
</tr>
<tr>
<td>MCP-1 (pg/mL)</td>
<td>196.95 ± 65.8</td>
<td>189.08 ± 140.6</td>
<td>192.4 ± 139</td>
<td>ns</td>
</tr>
<tr>
<td>IGF-1 (pg/mL)</td>
<td>295.58 ± 541.6</td>
<td>255.19 ± 381.7</td>
<td>205.12 ± 362.1</td>
<td>ns</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>1 ± 2</td>
<td>1 ± 1.9</td>
<td>1.8 ± 2.2</td>
<td>ns</td>
</tr>
</tbody>
</table>

Median ± IQR, ns – not significant, GSH – glutathione, GSSG – glutathione disulfide, 8OHdG – 8-hydroxy-2-deoxyguanosine, IL-1β – interleukin 1 beta, IL-6 – interleukin 6, IL-10 – interleukin 10, MCP-1 – monocyte chemo-attractant protein-1, IGF-1 – insulin like growth factor – 1, CRP – C reactive protein.
Figure 1. Medication usage chart comparison

![Medication usage chart comparison](image)

Dmeds – diabetic medication, Anti HT – antihypertensives, NSAID – non steroidal anti-inflammatory drugs

Figure 1 expresses medicated patients with each medication classification expressed as a percentage of the total for the respective BGL level.

Inflammatory markers expressed as ratios (Table 5) and the 1st and 4th, 5th quintiles were compared. IL-1β/IL-10, IL-6/IL-1β and CRP/IL-6 were significant at $p <0.05$

<table>
<thead>
<tr>
<th></th>
<th>Q 1*</th>
<th>Q 4,5*</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP/IL-10</td>
<td>0.08 ± 0.1</td>
<td>0.06 ± 0.2</td>
<td>ns</td>
</tr>
<tr>
<td>IL-6/IL-10</td>
<td>0.33 ± 1.7</td>
<td>0.4402 ± 0.9</td>
<td>ns</td>
</tr>
<tr>
<td>IL-1β/IL-10</td>
<td>0.09 ± 0.2</td>
<td>0.18 ± 0.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MCP-1/IL-10</td>
<td>7.19 ± 13.5</td>
<td>8.36 ± 12.4</td>
<td>ns</td>
</tr>
<tr>
<td>IGF-1/IL-10</td>
<td>6.83 ± 24.9</td>
<td>8.39 ± 14.3</td>
<td>ns</td>
</tr>
<tr>
<td>IGF-1/MCP-1</td>
<td>1.31 ± 1.5</td>
<td>1.48 ± 4.5</td>
<td>ns</td>
</tr>
<tr>
<td>IL-1β/CRP</td>
<td>1.59 ± 2</td>
<td>2.24 ± 6.3</td>
<td>ns</td>
</tr>
<tr>
<td>IL-6/IL-1β</td>
<td>5.71 ± 11.7</td>
<td>2.37 ± 9.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CRP/IL-6</td>
<td>0.45 ± 0.6</td>
<td>0.36 ± 0.4</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* Median ± IQR, CRP – c reactive protein, IL-10 – interleukin-10, IL-6 – interleukin-6, IL-1β – interleukin-1 beta, MCP-1 – monocyte chemo-attractant protein-1, IGF-1 – insulin like growth factor -1,
**Figure 2.** CVD, HT and T2DM status

![Bar chart showing CVD, HT and T2DM status](chart.png)

T2DM – Type 2 diabetes mellitus, CVD – cardiovascular disease, HT - hypertension

Figure 2 - Patients selected for this study attended the Charles Sturt University rural health diabetic screening program and were not discriminated on the basis of clinical or medication status. The results express the number of CVD, HT and T2DM patients as a percentage of the total for each respective group. The CVD, HT and T2DM group is the combined sum of patients with all 3 categories present

**Discussion**

This study has highlighted the value in incorporating emerging inflammatory and oxidative stress biomarkers as part of screening hyperglycaemic clients irrespective of any treatment regimen. Our results have replicated findings of well-controlled experiments comparing oxidative stress and inflammatory markers in diabetes progression, and demonstrated a substantive association with hyperglycaemia, oxidative stress and the inflammatory in a screening population (Al-Aubaidy & Jelinek, 2011a; Butkowski et al., 2016; Calabrese et al., 2012; Dinarello et al., 2010; van Exel et al., 2002).

Hyperglycaemia is associated with increased risk of T2DM associated with oxidative stress and an inflammatory process (de Carvalho et al., 2012). Significant differences were observed in WC, BMI, BGL, HbA1c, TG, HDL and TC/HDL between the normoglycaemic and elevated glycaemia groups (Table 2). These findings support their association with T2DM and affirms the hypothesis that traditional markers do assist in demonstrating that a continuum exists between chronic increasing hyperglycaemia and the progression to and development of T2DM. However traditional markers such
as HbA1c miss approximately 20% of people with diabetes if a cut-off of 6.5% is applied (Cowie et al., 2010)

The constellation of oxidative stress and inflammation factors emerging from and participating with the pro-inflammatory hyperglycaemic state is not fully understood, but of major concern is the progression of patients with elevated glucose to T2DM and the associated morbidity (Antonio Ceriello & Motz, 2004; de Rekeneire et al., 2006; Esposito & Giugliano, 2004). The results of this study indicate that both oxidative stress and inflammation provided significant results when groups were categorised with a BGL <4.5mmol/L and > 6.1mmol/L (Table 3), irrespective of any medication. The oxidative stress markers GSH, GSH:GSSG and 8OHDG showed significant results. GSH has previously been shown to demonstrate an association with IFG and oxidative stress and the link to diabetes (Al-Aubaidy & Jelinek, 2010; G. D. Lowe, 2006; Nwose et al., 2008a). It has been previously demonstrated that GSH:GSSG showed a significant difference between a control group and impaired fasting glucose (IFG), supporting the notion that early thiol related oxidative stress occurs (H. F. Jelinek et al., 2014). If this is the case, and as demonstrated in this study, the significant GSH, GSH:GSSG results when glucose levels were >6.1mmol/L, support the concept of monitoring oxidative stress during sustained hyperglycaemia. A consequence of this is an increased risk of micro and macrovascular complications (Sekhar et al., 2011) due to the increased ROS contributing to the structural and functional damage of endothelial cells and smooth muscle proliferation, a factor in the development of atherogenesis (Al-Aubaidy & Jelinek, 2011b; L. L. Wu, C. C. Chiou, P. Y. Chang, & J. T. Wu, 2004). 8OHDG is also considered an important biomarker for oxidative stress in diabetes progression and indicator of the pre-diabetic state (Al-Aubaidy & Jelinek, 2010). However previous findings have disputed a link between hyperglycaemia and oxidative stress (Choi, Benzie, Ma, Strain, & Hannigan, 2008), with positive and negative associations which have been linked to lipid levels (Kikuchi et al., 2013; Miyamoto, Kotani, Ishibashi, & Taniguchi, 2011; Subash, Gurumurthy, Sarasabharathi, & Cherian, 2010). In this study lipid studies did show a significant difference between glucose levels <4.5mmol/L and levels >6.1mmol/L. As we did not discriminate in this study between disease and the medication state the
oxidative stress markers supply us with an important element, along with inflammatory markers in their utility in screening hyperglycaemic patients.

Elevated levels of the pro inflammatory IL-1β has been associated with hyperglycaemia, insulin resistance, obesity, all factors which contribute to the development of T2DM (Koenen et al., 2011). The current confirmed that a significant increase of IL-1β is associated with hyperglycaemia and supports previous findings of elevated IL-1β in T2DM, possibly as a result of chronic inflammation and insulin resistance (Stentz, Umpierrez, Cuervo, & Kitabchi, 2004). Studies have shown that the neutralization of IL-1β assists in decreasing the inflammatory response in autoimmune, malignancy and other common disease states such as T2DM (Dinarello, 2011; Osborn et al., 2008). If so knowledge of IL-1β levels may provide an indication of earlier treatment intervention to allay the development of progression to T2DM and CVD in hyperglycaemia. Elevations of IL-6 have been demonstrated in hyperglycaemia/hyperinsulinaemia (Fishel, Watson, Montine, & et al., 2005; Morohoshi, Fujisawa, Uchimuraa, & Numano, 1996). We have previously demonstrated increased levels of IL-6 in T2DM (Butkowski et al., 2016). The anti-inflammatory marker IL-10 in this study was not significantly different between the two glucose groups. IL-10 is considered a potent anti-inflammatory, however mixed results have been observed (Straczkowski, Kowalska, Nikolajuk, Krukowska, & Gorska, 2005), Esposito has pointed out that a low innate production capacity of IL-10 may help identify subjects at more risk for inflammation in MS (Esposito & Giugliano, 2004; Esposito et al., 2003). Drug therapy to either control T2DM or prevent development is required if diet and lifestyle improvements are unable to modify and improve the risk factors resulting from hyperglycaemia. Whilst the anti DM, anti HT and statin use is much higher in glucose group (>6.1mmol/L), the use of NSAIDS was also heavily utilised in the low glucose (<4.5mmol/L) group (Fig 1). This may exert an effect on the anti-inflammatory effects of IL-10, however it has been shown that statins not only reduce cholesterol and TG but can independently decrease CRP levels (Averna & Lo Verde, 2003).

Whilst IL-1β was the only individual inflammatory marker demonstrating a significant difference, a ratio test of the emerging biomarkers, with CRP included, was tested for any degree of significance (Table 5). A previous
study into prognostic inflammatory outcomes did reveal an association of IL6/IL10 in systemic inflammatory response (Taniguchi et al., 1999). Studies on inflammatory markers conducted by Spranger noted that participants with a combined elevation of both IL-6 and IL-1β had about a three-fold increase in risk of developing diabetes, whereas low levels of IL-1β alone demonstrated no substantial increase in risk (Spranger et al., 2003). The current work also recognised the importance that multiple inflammatory markers may provide useful information in diagnosing the risk of DM progression. Significant results were obtained with IL-1/IL-10, IL-6/IL-1β, and CRP/IL-6. Studies reviewing IL-6 and tumor necrosis factor–α (TNF-α) have established roles in the regulation of APR’s with other investigations providing a possible link with these two biomarkers and cardiovascular events (Biswas, Ghoshal, Mandal, & Mandal, 2010), however inflammatory marker ratios and any association with hyperglycaemia has not been fully investigated. IL-6/IL-10 has been shown to decrease significantly in post-operative alcoholic patients with infections (Sander et al., 2002). It has been previously established that the anti-inflammatory IL-10 is induced by pro-inflammatory cytokines, thereby affording some protection in states of inflammation (Weis et al., 2009). Our observations showed that IL-1β/IL-10 was significant, whereas IL-10 did not increase compared to the control group. This could be explained by IL-10 not responding to small increases in BGL. However the pro-inflammatory IL-6/IL-1β and CRP/IL-6 ratios were significant whilst the biomarkers tested as stand-alone: IL-6 and CRP were not. IL-1β has been shown to be increased in T2DM (Dinarello et al., 2010), however there is a caveat as Zhao has demonstrated that IL-1β and its interaction with functional β-cell mass leading to overt T2DM may not be reversible by glucose lowering therapy (Zhao, Dharmadhikari, Maedler, & Meyer-Hermann, 2014). This further highlights and supports this study that inflammatory cytokine and oxidative stress ratios may provide additional information on progressive hyperglycaemia in a screening cohort.

**Conclusion**

Our current results support previous findings that inflammation and oxidative stress are associated with increased blood glucose levels and suggest that
GSH, GSH:GSSG, 8OHDG and IL-1β may play a role in aiding clinicians in identifying hyperglycaemic patients with inflammatory and oxidative stress pathology and risk of developing T2DM and CVD. Importantly are our findings demonstrating significant difference observed in inflammatory and oxidative stress marker ratios. To our knowledge this is the first time ratio analysis of these markers in hyperglycaemic patients has been undertaken. Using anthropomorphomeric, traditional markers and emerging marker’s and/or ratios in hyperglycaemic management may provide valuable information leading to more preventative measures at a clinical level. As these novel ratio findings are largely untested in the hyperglycaemic state further investigations are required to support our findings.

**CONFLICT OF INTEREST**

**THE AUTHOR(S) DECLARE(S) THAT THERE IS NO CONFLICT OF INTEREST REGARDING THE PUBLICATION OF THIS ARTICLE**

**ACKNOWLEDGEMENT**

Roche Australia provided the glucose measuring sticks and glucometers. Bev de Jong provided technical assistance Simon McDonald from SPAN assisted in the statistical analysis.

**References**


Miyamoto, M., Kotani, K., Ishibashi, S., & Taniguchi, N. (2011). The relationship between urinary 8-hydroxydeoxyguanosine and


Chapter 6

Paper 5: Antidiabetic, antihypertensive and statin medication use in metabolic syndrome


In this paper I initiated the current literature search and write-up to investigate medication use in outpatient communities. Ms L Brix, a science intern from Germany assisted in the data analysis of this publication as part of her internship studies at Charles Sturt University. Dr’s Al-Aubaidy, Jelinek and Professor Kiat provided direction, critique, and contributed to the introduction and discussion. All authors approved the final submission for publication.

From our previous paper we observed that results obtained for certain biomarkers in a screening population replicated well controlled experimental results. This supported the potential use of certain biomarkers as a screening tool for hyperglycaemic patients in outpatient clinics. The prediabetic state, evidenced by the presence of hyperglycaemia is considered to be one of the most important factors in the progression to T2DM.

Patients were classified as metabolic syndrome (MetS) if 3 or more factors according to the National Cholesterol Education Program Adult Treatment Panel III (ATP III) were present. Patients with MetS have a fivefold increased risk of T2DM and a twofold increased risk of developing atherosclerotic CVD. A common finding and independent diagnostic criterion for MetS is the presence of hyperglycaemia, therefore intensive glycaemic control may
provide cardiovascular benefit for early T2DM. If insulin resistance is a risk factor in MetS, improving glucose control and insulin resistance through medication may be another target to consider (in addition to physical activity). Antidiabetic (Dmeds), antihypertensive (HT) and Statin use differed significantly between No MetS and MetS groups. For example Dmeds tripled ($p < 0.001$), which was the biggest increase in medication type use. This study did show that medication did increase with ATPIII, however participants with a FBG >6.1mmol/L were not found to have Dmeds prescribed; whereas HT and statins were extensively utilised when only 1 or 2 MetS factors were present. In addition we showed Statin use may also be below that recommended as the increased systolic blood pressure and HT category was quite high suggesting there is a higher risk of CVD in this population. Biomarkers were not included in this study however the findings would support a study of emerging biomarkers in MetS patients with the inclusion of the medication categories discussed in this paper.

As we continued our studies into the hyperglycaemic state we next investigated the role of homocysteine, a marker for endothelial dysfunction, and any contributory role it may have in the MetS.
Antidiabetic, antihypertensive and statin medication use in metabolic syndrome

Butkowski, E.\textsuperscript{1}, Brix, L.\textsuperscript{1,2}, Al-Aubaidy, H.A.\textsuperscript{3}, Kiat, H.\textsuperscript{4} and Jelinek, H.F.\textsuperscript{1,4}

\textsuperscript{1} School of Community Health, Charles Sturt University, Albury, Australia.
\textsuperscript{2} Department of Biology, Ludwig-Maximilians-University, Munich, Germany.
\textsuperscript{3} School of Medicine, University of Tasmania, Hobart, Australia.
\textsuperscript{4} School of Medicine, University of New South Wales and Faculty of Medicine and Health Sciences, Macquarie University, Sydney, Australia

Address for Correspondence
Herbert F. Jelinek
School of Community Health
Charles Sturt University
Albury 2640
Australia
E: hjelinek@csu.edu.au

Keywords
Metabolic Syndrome; medication; antihypertensives; statins; antidiabetic; prevalence; community health.

Abstract

\textbf{Background:} Metabolic syndrome (MetS) characterised by a cluster of metabolic risk factors, which eventually increase the risk of diabetes and cardiovascular disease. The aim of the current study was to investigate medication use in outpatient communities with respect to the occurrence of these metabolic risk factors as defined by ATPIII.

\textbf{Methods:} Data for this study was obtained from patients attending a diabetes health screening clinic (DiabHealth) in south-eastern Australia between 2005 and 2012. Participants had a medical history taken and anthropomorphic data collected. Participants with three or more MetS factors were classified as MetS positive as outlined by the National Cholesterol Education Program Adult Treatment Panel III (ATP III).
Results: Antidiabetic, antihypertensive and antihyperlipidaemic use varies significantly in uptake by participants and with respect to the number of ATPIII factors present. Blood glucose levels (BGL) and the female waist circumference were significantly better in the MetS compared to the non-MetS group. The most increase in medication use in the MetS group was seen for antidiabetic medication (21.3% versus 2.4%, p < 0.01) compared to the non-MetS group. Antihypertensive use tripled (67.8% vs. 26.03%) and Statin use doubled significantly (p<0.01) in the MetS group (21.8% vs. 8.9%).

Conclusion: Medication use increases with an increase in ATPIII factors present in the study. Participants with increased BGL (>6.1 mmol/L) were not found to have antihyperglycemic medication prescribed. However both antihypertensive medication and Statins were extensively prescribed in cases where only 1 and 2 ATP factors for MetS were present.

Introduction

The Metabolic Syndrome – Definition and Prevalence

The metabolic syndrome (MetS) is a cluster of metabolic risk factors associated with a 5-fold increased risk of type 2 diabetes (T2DM) and a 2-fold increased risk of atherosclerotic cardiovascular disease. The National Cholesterol Education Program Adult Treatment Panel III (ATP III)’s defined a set of to identify patients having the MetS and viewed CVD as the primary clinical outcome of this disease (P. L. Huang, 2009; C. K. Roberts, Hevener, & Barnard, 2013). The 5 criteria identified by the ATPIII of which the presence of any three or more comprise the MetS is listed in Table 1 (S. M. Grundy, H. B. Brewer, Jr., J. I. Cleeman, S. C. Smith, Jr., & C. Lenfant, 2004).
Table 1: ATP III Modified Clinical Identification of the Metabolic Syndrome

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Defining Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference (cm):</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>&gt; 102</td>
</tr>
<tr>
<td>Women</td>
<td>&gt; 88</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>≥ 1.7</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)*</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>&lt; 1.04</td>
</tr>
<tr>
<td>Women</td>
<td>&lt; 1.30</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>≥ 130/ ≥ 85</td>
</tr>
<tr>
<td>Fasting Blood Glucose (mmol/L)</td>
<td>≥ 6.1</td>
</tr>
<tr>
<td>Use of antidiabetic, antihypertensive or Statin medication</td>
<td></td>
</tr>
</tbody>
</table>

*HDL – high density lipoprotein

Whilst insulin resistance is not a required criterion for MetS using the ATPIII classification, the presence of T2DM or antihyperglycemic medication is considered in its diagnosis (S. M. Grundy, H. B. Brewer, Jr., et al., 2004). Additional definitions have been recommended by the World Health Organization (WHO), American Association of Clinical Endocrinologists and the International Diabetes Federation (IDF) (Scott M. Grundy et al., 2004) (K. G. M. M. Alberti, Zimmet, & Shaw, 2005). Whilst there are some important differences in ranking of the predominant causative factors, there is recognition of similar criteria to the ATPIII definition of MetS. However, a major difference between the definition of the ATPIII and the IDF is the latter does include the patient’s medication as a criterion for the MetS. This additional criterion does allow either BGL or triglycerides to be in the normal range.

One of the most important risk factors leading to T2DM is the presence of prediabetes. Prediabetes is defined by either an impaired fasting (BGL > 6.1mmol/L) or post-prandial blood glucose level (BGL > 11mmol/L). Together with other potential risk factors for CVD, according to the ATPIII classification prediabetes is a major cause of the metabolic syndrome and one
of its defining factors (S. M. Grundy, 2012). Additional underlying metabolic risk factors such as obesity and abnormal body fat distribution account for 20% and 30% of the adult population and predispose to MetS (S. M. Grundy, 2007, 2008). Although not included in the ATPIII classification, age correlates positively with MetS (Bulhöes & Araujo, 2007).

**MetS and Glucose Lowering Medication**

A common finding and independent diagnostic criterion for MetS is the presence of hyperglycaemia. Whilst the major studies conducted in 2008 and 2009 - UK Prospective Diabetes Study (UKPDS), Veteran’s Affairs Diabetes Trial (VADT), Action to Control Cardiovascular Risk in Diabetes (ACCORD), Action in Diabetes and Vascular Disease (ADVANCE) and Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycaemia in Diabetes (RECORD) reviewed the extensive use of antiglycaemic agents as primary strategies in the treatment for MetS, the need for more intensive glycaemic control may also provide cardiovascular benefit for early T2DM with no demonstrated presence of atherosclerosis (Pelikanova, 2009). If insulin resistance is one of the risk factor in MetS, improving glucose control and insulin resistance through pharmaceutical agents will be another target to be considered in addition to physical activity. However, some drug strategies such as use of metformin for improving insulin resistance are not routinely used for decreasing risk of T2DM and CVD if prediabetes is present (Scott M. Grundy et al., 2004). Mixed results have been reported on whether antihyperglycaemic medication decreases CVD risk in patients with prediabetes or T2DM. Only empagliflozin has been shown to improve cardiovascular prognosis. In a recent prospective study, cardiovascular events have not increased with insulin treatment with or without metformin (Ferrannini & DeFronzo, 2015; Hinnen, 2015).

**Antihypertensive Medication and MetS**

Antihypertensive drug treatment is recommended for MetS patients when BP is >140/90mmHg. Observations from the Framingham Risk Study (FRS) state that vascular disorders are central to MetS as indicated that 80% of men and up to 65% of women with hypertension are obese (Garrison, Kannel, Stokes, & Castelli, 1987). Insulin resistance has been associated with the
development of HT, possibly through a variety of mechanisms involving sodium imbalance, imbalance between the release of nitrous oxide and endothelin-1, insulin action, adipokine activity due to increased adipose tissue and obesity (including perivascular adipose tissue and vascular function), decreased levels of adiponectin, adipokine activity and increased tumour necrosis factor α (TNF-α) (Mendizbal, Llorens, & Nava, 2013). Additionally the importance of genetics cannot be underestimated. Hopkins and Hunt (2003), have provided an extensive review of genetic markers that may contribute to the development of HT (Hopkins & Hunt, 2003). Whilst genetic analysis is still somewhat impractical and economically prohibitive as a diagnostic screening tool, as technology continues to improve these costs will come down.

**MetS and Statins**

The statins are a class of drugs which act to lower total cholesterol and LDL levels by reducing hepatic cholesterol production through inhibition of hydroxyl methyl glutamyl Co-A (HMG-CoA) reductase and a reduced CVD incidence (Ebrahim, Taylor, & Brindle, 2014). Statins are also known to reduce circulating triglyceride levels (Isley, Miles, Patterson, & Harris, 2006). 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 (S. Sandhu, Wiebe, Fried, & Tonelli, 2006). As MetS may progress to T2DM and increased CVD it should be considered to be an inflammatory state. The use of statins has been shown to decrease circulating levels of C Reactive protein (CRP), independently to its lipid lowering effect (Averna & Lo Verde, 2003).

The successful treatment of MetS involves addressing all of the risk factors treatment regimes. Whilst lifestyle and diet has emerged as a major preventative approach, these changes alone may not control or prevent the development of the risk factors categorising MetS. The current study investigated the use of antihyperglycemic, antihypertensive and lipid lowering (statins) drugs and their associated use in MetS and how medication use differs with respect to the number of MetS factors identified.
Materials and Methods

Data for this study was obtained from patients attending a diabetes health screening clinic (DiabHealth) in south-eastern Australia between 2005 and 2012. Participants were recruited via public media announcements. The screening and data collection were carried out within the School of Community Health at Charles Sturt University (CSU). Participants had a medical history taken and anthropometric data collected in addition to screening for MetS factors. Thresholds for MetS criteria were taken from the definition of the National Cholesterol Education Program Adult Treatment Panel III (ATP III) (see Table 1). Participants who met three or more criteria were classified as MetS positive. In the current study, medication use was also taken into account in classifying patients into the No MetS or MetS group as described in the definition of the International Diabetes Federation (IDF).

Age, gender, body mass index (BMI) (low <20 kg/m², normal <25 kg/m², overweight 25–30 kg/m², and obese >30 kg/m² and waist circumference (measured at the midpoint between the lower border of the rib cage and the iliac crest by using a flexible inch tape) were obtained. Blood pressure (BP) measurements were taken using a standard mercury sphygmomanometer and a cuff of appropriate size after the individual had rested for at least five minutes in a supine position. BP was recorded in a sitting position in five individuals with the arm supported at heart height, as this was more comfortable for these five patients. A comprehensive list of prescription medications was provided by each patient. Medication profile for each participant was collected and data sorted into antihypertensive, statin and antidiabetic use.

The data was analysed using R statistical computing (Version 3.2.3 for Windows) and Microsoft Excel (Office2007, Microsoft). All values were expressed as mean ± standard deviation (M±SD). Statistical analysis was performed using an independent sample t-test for two group comparisons of continuous normally distributed data or Chi-square statistics were used to investigate categorical data. In addition proportions analysis was used to compare the data between the five MetS factors. Post-hoc pairwise comparisons were performed using the Benjamini and Yekutieli correction after significant effects were found following the proportions test (Benjamini
& Yekutieli, 2001). ANOVA and post hoc statistics was applied for clinical continuous multigroup data. In all tests, p < 0.05 was considered to be statistically significant. Power analysis was based on a median effect size and high power, suggesting a sample number of 27 with a p value of 0.05 to be sufficient to establish meaningful differences (Kirby, Gebski, & Keech, 2002).

Results

During the screening period from January 2005 to October 2011, 1614 volunteers attended the Diabetes Health (DiabHealth) clinic at CSU, Albury (H.F. Jelinek, Wilding, & Tinley, 2006). Excluding repeat visits, 531 participants had complete data, which was analysed for demographic and clinical attributes, and the five factors of the MetS.

Table 2: ATPIII factors of the study population

<table>
<thead>
<tr>
<th></th>
<th>MetS</th>
<th>No MetS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC_Females*</td>
<td>88.8 ±13</td>
<td>94 ± 14.3</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>WC_Males</td>
<td>101.5 ± 13.2</td>
<td>103.8 ± 11.7</td>
<td>ns</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>130.7 ± 17.3</td>
<td>131.5 ± 17.4</td>
<td>ns</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>76.1 ± 8.8</td>
<td>76.3 ± 9.1</td>
<td>ns</td>
</tr>
<tr>
<td>HDL_Females</td>
<td>1.54 ± 0.4</td>
<td>1.5 ± 0.4</td>
<td>ns</td>
</tr>
<tr>
<td>HDL_Males</td>
<td>1.33 ± 0.6</td>
<td>1.2 ± 0.3</td>
<td>ns</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.47 ± 0.9</td>
<td>1.42 ± 0.8</td>
<td>ns</td>
</tr>
<tr>
<td>BGL (mmol/L)</td>
<td>5.5 ± 1.7</td>
<td>5.8 ± 1.8</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

* WC - waist circumference; SBP – systolic blood pressure; DBP – diastolic blood pressure; HDL – high density lipoprotein; BGL – blood glucose levels; mean ± standard deviation; ns - non significant.

Waist circumference for males and SBP/DBP were not significantly different between the two groups (Table 2). Only WC for females was significantly higher in the No MetS group compared to the group with MetS (3 or more of the five factors) (Table 1). Biomarker analysis indicated that HDL, triglycerides and BGL were within recommended limits. BGL was significantly higher (p < 0.05) in the No MetS group but still below the cut-off of 6.1mmol/L (Table 1).
Participants were screened for their medication in context with MetS. Groups were divided into no MetS (0–2 factors) and MetS (3-5 factors). Of 531 patients attending the Diab Health screening 70 were clear of any MetS factors and were receiving no antidiabetic, antihypertensive or statin medication.

**Table 3: Percentage use of medication for patients with and without metabolic syndrome**

<table>
<thead>
<tr>
<th>Medication</th>
<th>MetS* (n=239)</th>
<th>NoMetS (n=292)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Diabetes</td>
<td>51</td>
<td>7</td>
<td>2.4</td>
</tr>
<tr>
<td>Anti-Hypertension</td>
<td>162</td>
<td>76</td>
<td>26.03</td>
</tr>
<tr>
<td>Statins</td>
<td>52</td>
<td>26</td>
<td>8.9</td>
</tr>
</tbody>
</table>

*MetS – Metabolic Syndrome present (Factors ≥ 3); NoMetS – Metabolic Syndrome not present (Factors < 3); (%) – percentage

Antidiabetic, antihypertensive and Statin use combined differed significantly between the MetS and No MetS groups (p < 0.0001). When medication use was separated into anti-diabetic, anti-hypertensive and Statins, similar significant differences was found between the MetS and No MetS groups (Table 3).

In the following tables (Tables 4-6) medication use with respect to presence of MetS factors is shown for antidiabetes (Dmeds) and antihypertension (anti-HT) medication and Statins.

**Table 4: Number of participants using diabetes medication for each MetS factor**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Dmeds*</th>
<th>Total N</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>106</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>116</td>
<td>5.2</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>132</td>
<td>15.9</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>80</td>
<td>22.5</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>27</td>
<td>44.4</td>
</tr>
</tbody>
</table>

*Dmeds – number of patients on one or more antidiabetic medication

Antidiabetic medication use tripled (p < 0.001) when going from No MetS (< 3 factors) to MetS (≥ 3 factors). Comparing medication use with respect to number of ATPIII factors present indicated significant differences (p < 0.001) between medication use and the number of ATPIII factors present except between 1 and 2, 2 and 3, 3 and 4, and 4 and 5 factors present (Table 4).
**Table 5: Number of participants using anti-hypertension medication for each MetS factor**

<table>
<thead>
<tr>
<th>Factor</th>
<th>anti-HT*</th>
<th>Total N</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>106</td>
<td>20.6</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>116</td>
<td>46.6</td>
</tr>
<tr>
<td>3</td>
<td>81</td>
<td>132</td>
<td>61.4</td>
</tr>
<tr>
<td>4</td>
<td>59</td>
<td>80</td>
<td>73.8</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>27</td>
<td>81.5</td>
</tr>
</tbody>
</table>

*Anti-HT – Antihypertensive medication*

Anti-hypertensive medication also increased significantly with the number of factors considered (p < 0.001). However no significant increases were noted when the number of ATPIII factors increased from 3 to 5 (Table 5).

**Table 6: Number of participants using statins for each MetS factor**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Statins</th>
<th>Total N</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>106</td>
<td>3.8</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>116</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>132</td>
<td>21.2</td>
</tr>
<tr>
<td>4</td>
<td>19</td>
<td>80</td>
<td>23.8</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>27</td>
<td>18.5</td>
</tr>
</tbody>
</table>

Statin usage increased with the number of MetS factors present. Significant differences in statin use with respect to number of factors present (Table 6) was seen for all comparisons (p < 0.0001) except when comparing between 2 and 3, 3 and 4 and between 4 and 5 factors present (Table 6).
Comparison of antidiabetic agents, antihypertension medication and Statins in Figure 1 indicates that the antihypertensive class is more prescribed in association with all categories. In general statin use is less prescribed in this population than diabetes in the MetS group (≥ 3 factors). The use of antidiabetic agents steadily increases and is similar to the antihypertensives once five factors of MetS are present in the patients. Effectiveness of treatment with respect to MetS factors is shown for the MetS factors indicated by ATPIII (Table 1).

Table 7. ATPIII biomarker levels with respect to number of MetS factors

<table>
<thead>
<tr>
<th>Factors</th>
<th>WCF (cm)</th>
<th>WCM (cm)</th>
<th>Trigs (mmol/L)</th>
<th>HDLF (mmol/L)</th>
<th>HDLM (mmol/L)</th>
<th>SBP (mmHg)</th>
<th>BGL (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96 ± 11.5</td>
<td>104.1 ± 13.3</td>
<td>1.41 ± 0.7</td>
<td>1.6 ± 0.4</td>
<td>1.55 ± 0.4</td>
<td>130.9 ± 17.6</td>
<td>6.3 ± 2</td>
</tr>
<tr>
<td>2</td>
<td>92.4 ± 16.6</td>
<td>103.4 ± 11.3</td>
<td>1.41 ± 0.9</td>
<td>1.46 ± 0.4</td>
<td>1.47 ± 0.4</td>
<td>131.7 ± 17.4</td>
<td>5.5 ± 1.5</td>
</tr>
<tr>
<td>3</td>
<td>88.5 ± 12.7</td>
<td>101.3 ± 13</td>
<td>1.46 ± 1</td>
<td>1.52 ± 0.4</td>
<td>1.51 ± 0.4</td>
<td>131.1 ± 17.7</td>
<td>5.6 ± 1.7</td>
</tr>
<tr>
<td>4</td>
<td>89.4 ± 14.4</td>
<td>98.2 ± 12</td>
<td>1.26 ± 0.6</td>
<td>1.6 ± 0.5</td>
<td>1.65 ± 0.4</td>
<td>124.3 ± 14.5</td>
<td>5 ± 1.1</td>
</tr>
<tr>
<td>5</td>
<td>91.3 ± 14.9</td>
<td>111.5 ± 19.7</td>
<td>2.1 ± 1.6</td>
<td>1.58 ± 0.2</td>
<td>1.47 ± 0.3</td>
<td>142.3 ± 11.7</td>
<td>6 ± 2.6</td>
</tr>
</tbody>
</table>

WCF – waist circumference females, WCM – waist circumference males, Trigs – triglycerides, HDLF – high density lipoproteins females, HDLM – high density lipoprotein males, SBP – systolic blood pressure, BGL – blood glucose
The only significant difference observed was for waist circumference in females when between 1 and 3 MetS factors were present (p<0.01). However waist circumference for females was at best borderline, observed when 3 ATP III factors were present and was highest for the group with one MetS factor. Similarly waist circumference for males was only within the desired level if three or four ATP III factors were present. All other differences in biomarker levels associated with the number of ATP III factors present showed no significant differences. Triglyceride levels for 5 factors was above the desirable level of <1.7mmol/l (Scott M. Grundy, 2012). For HDL the desirable levels for females are >1.3 mmol/L and for males >1.04 mmol/L. Only the group with any one of the ATP III factors present had an elevated mean BGL value (Table 7).

Discussion

ATP III criteria for diagnosis of MetS are practical to use in a clinical setting. According to ATP III the presence of any three factors (Table 1) constitutes MetS (S. M. Grundy et al., 2005). Management of MetS must first start by addressing factors that are modifiable such as smoking, alcohol use and lack of physical exercise. Prophylactic use of medications such as Statins may also be warranted even on patients with normal cholesterol levels suggested by outcomes from the Heart Protection Study Collaboration (HPS) and the Collaborative Atorvastatin Diabetes Study (CARDS) (Colhoun et al., 2004; "MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 5963 high-risk individuals: a randomised placebocontrolled trial," 2003). The most widely recognized of the metabolic risk factors associated with metabolic syndrome are high cholesterol, hypertension, and elevated blood glucose levels. Depending on which factors are present MetS increases the risk of overt diabetes and cardiovascular disease (Rydén et al., 2007). Drug therapy is essential if modifiable risk factors such as lifestyle practices and diet are not controlling abnormal levels of BGL, systolic blood pressure and cholesterol. The current study investigated the use of medication reported by patients attending a diabetes health screening clinic (DiabHealth) and the presence of single and multiple MetS factors. The study classified MetS as the presence of any three factors of five present as defined by the ATP III classification system but included the use of medication for raised BGL,
blood pressure and LDL as an additional criterion as suggested by the *IDF* classification.

The biggest increase of medication when comparing MetS to No MetS (< 3 factors vs ≥ 3 factors) was seen for antidiabetic medication use suggesting that incidence of diabetes may be strongly related to the increase in obesity, blood pressure and cholesterol levels as observed in this study where the mean waist circumference was elevated in the nonMetS group and remained borderline in the MetS group. BGL was significantly different between the two groups but was lower in the MetS group possibly associated with the increase in patients with T2DM and the associated use of antidiabetic medication in the MetS group (Table 3) (Scott M. Grundy, 2012). Analysis of medication use with respect to the number of MetS factors present indicated that there was no significant increase between any of the biomarkers. Antidiabetic medication use trebled between 2 and 3 MetS factors present and the most significant difference was observed between one and five MetS factors present. This reflects the importance of dealing with hyperglycaemia following the Insulin Resistance Atherosclerosis Study (IRAS), which reported a nearly five times greater risk of coronary artery disease for the group with the lowest insulin sensitivity (C. K. Roberts et al., 2013)

Antihypertensive medication was the most often prescribed group (31.1%) compared to the antidiabetic (6.4%) and Statins (19.5%) if only 1 MetS factor of the possible five was present (Table 1). SBP was borderline as recommended by *ATPIII* for factors 0 to 3. A dramatic increase in the mean of SBP to above 140mmHg was seen in association with 4 MetS factors present, which then dropped to ideal levels when 5 factors were present. This result reflects the biomarker levels reported with no significant difference between the No MetS
group and MetS group for CVD risk factors apart from waist circumference (p < 0.001), which decreased significantly below the MetS cut-off in the MetS group. The cholesterol biomarkers were all within normal limits. Disparities in our study with medication use are associated with our nonspecific categorisation of the MetS characteristics where the presence of one factor can be any one of the five and the presence of three or more the combination of any of the five factors defined by ATPIII. Table 7 indicates that only waist circumference is above the cut-off value recommended by ATPIII. However SBP and cholesterol levels are below the cut-off due to the use of antihypertensive and statin use. This suggests that preventative measures are having an effect on preclinical MetS (<3 factors present) and BGL, blood pressure and total cholesterol and HDL are controlled in the MetS patient group, which show levels lower than those found in the non-MetS group. Medication use increases with an increase in ATPIII factors present in the study. However participants with increased BGL (>6.1mmol/L) were not found to have antihyperglycemic medication prescribed. Both antihypertensive medication and statins were extensively prescribed in cases where only 1 and 2 ATPIII factors for MetS were present. Several limitations of the study have to be noted including the self-reporting of medication use and the associated compliance by participants is not verified. In addition confounding factors may play a role in medication use, especially in the non-MetS group who may have only one or two MetS factors present such as economic status and education level.

Findings in our study indicates that in the focused community of outpatients the metabolic syndrome is relatively well controlled and the majority of the risk factors for CVD are below the documented threshold level. However, waist circumference remains higher than recommended, suggesting that lifestyle practices may need to be addressed more to achieve an optimum response to the treatment (Kaur, 2014a). Statin use may also be below that recommended as the increased SBP and antihypertensive medication use category is quite high (Figure 1) suggesting that there is a high risk of CVD in this population.
CONFLICT OF INTEREST

THE AUTHOR(S) DECLARE(S) THAT THERE IS NO CONFLICT OF INTEREST REGARDING THE PUBLICATION OF THIS ARTICLE

ACKNOWLEDGEMENT

Roche Australia provided the glucose measuring sticks and glucometers. Bev deJong provided technical support.

References


In this paper the role of homocysteine (Hcy), glutathione (GSH) and 8-hydroxy-2’- deoxyguanosine (8-OHdG) in the progression to metabolic syndrome (MetS) was examined. Medication was included to enable a population study to determine whether changes in Hcy were usable as T2DM and CVD risk indicators in community health screening.

My role in this study was in the development of the research question and organisation and collection of blood samples at South West Pathology (SWPS). I supervised and conducted the assays for tests performed at SWPS. Testing included lipid studies, HbA1c, glucose, CRP and homocysteine. The emerging biomarkers IL-6, MDA, GSH and 8-OHdG were assayed at Charles Sturt University. I undertook responsibility for the design of the experimental procedure and collection and correlation of results. Dr’s Jelinek and Al-Aubaidy assisted me in the compilation of the article and provided me with valuable input and direction. Each author approved the final drafting prior to submission for publication.

In furtherance of our studies on oxidative stress in hyperglycaemia we investigated the role Hcy, a promoter of atherosclerosis through increased oxidative stress, impaired endothelial function and induction of thrombosis.
Hcy is a link between OS and the inflammatory process. In this study the glucose lowering, antihypertensive and statin use was included to enable the examination of the utility of Hcy as a usable indicator of T2DM and CVD risk. Significantly elevated results for 8-OHdG between 2 MetS and 3 MetS factors (p <0.001) was observed. CRP rose between 0 MetS vs 1 MetS and 3 MetS (p <0.05). Hcy increased steadily with significant differences between no MetS and 3 MetS (p=0.001) and 4 MetS (p=0.007) as well as 1 MetS and 3 MetS (p=0.013) and 2 MetS vs 3 MetS (p=0.013) and 2 MetS vs 3 MetS (p=0.034). Since the definition of MetS does not consider factors such as age, gender, LDL and emerging OS, inflammatory or coagulation biomarkers. Endothelial dysfunction being a hallmark of CVD and diabetes requires a clinically assessable biomarker such as Hcy. Our analysis confirms that GSH and 8-OHdG may also be useful in MetS investigations, as both have been shown to be associated with hyperglycaemia.

Our conclusion indicated that the significant results associated with Hcy and clustering of MetS factors may be the result of complex pathophysiological metabolic interactions with OS markers, suggesting vascular pathology increases in concordance with an increase in MetS factors.

Paper 7 investigated a proposal that low levels of creatinine may be useful as a marker of prediabetes. We were able to test this thesis on archivable data.
Interaction of homocysteine, glutathione and 8-hydroxy-2´-deoxyguanosine in metabolic syndrome progression

Butkowski, E.G.¹ Al-Aubaidy, H.A.² and Jelinek, H.F.¹,³*

¹ School of Community Health, Charles Sturt University, Albury, Australia.
² School of Medicine, University of Tasmania, Hobart, Australia.
³ Australian School of Advanced Medicine, Macquarie University, Sydney, Australia.

*Corresponding author.
Herbert Jelinek
School of Community Health
Charles Sturt University
Albury,
Australia
T:+61 2 60519219
E: hjelinek@csu.edu.au

Abstract

Purpose: The role of homocysteine (Hcy) and associated oxidative stress processes in the metabolic syndrome (MetS) continuum has not been explored extensively. This paper explores changes in Hcy and associated oxidative stress in relation to the number of metabolic syndrome factors present

Method: Participants at the rural diabetes screening clinic had their medical history recorded as well as Hcy, body mass index, blood glucose levels, cholesterol, glutathione (GSH), and 8-hydroxy-2-deoxyguanosine (8-OHdG) measured.

Result: A significant elevation in Hcy (9.47±2.9 vs. 10.56±3.3, p=0.03) and 8-OHdG (307.7±516 vs. 1130.6±1155, p=0.0001) was observed between the noMetS and MetS groups. Hcy steadily increased with the addition of MetS factors paralleled by 8-OHdG and glutathione (GSH). The most dramatic increase was seen in 8-OHdG, which nearly doubled between 2 MetS and 3 MetS factors present (p=0.0001).

Conclusion: Homocysteine may be a useful marker together with 8-OHdG in assessing the extent of metabolic syndrome

Keywords: Homocysteine, 8-OHdG, glutathione, metabolic syndrome, oxidative stress,
Abbreviations:

BMI – Body Mass Index
CRP – C - reactive protein
CVD – cardiovascular disease
DBP – Diastolic blood pressure
GSH – Glutathione
GSSG – Glutathione disulfide
HbA1c – Glycated hemoglobin
Hcy – Homocysteine
HDL – High density lipoprotein
IGF-1 - Insulin-like growth factor-1
IL – Interleukin
LDL – Low density lipoprotein
MCP-1 - Monocyte Chemotactic Protein-1
MDA – Malondialdehyde
MetHb - Methemoglobin
MetS – Metabolic syndrome
8-OHdG – 8-hydroxy-2´-deoxyguanosine
PGI_{2} – Prostaglandin-I_{2}
T2DM – Type 2 diabetes mellitus
TC – Total cholesterol
TNF-α - Tumor necrosis factor-α
TG – Triglycerides
SBP – Systolic blood pressure
WC – Waist circumference

Introduction

Metabolic syndrome can be defined by clustering of fasting hyperglycemia, decreased HDL-Cholesterol, hypertriglyceridemia, increased WC and hypertension according to the National Cholesterol Education Program (NCEP) and Adult Treatment Program III (ATP III) (Scott M. Grundy, 2006). The clustering of MetS factors and the associated increased risk of diabetes and cardiovascular disease are a major concern for global health (Hotamisligil, 2006). A major shortcoming of current definitions of MetS is the lack of inclusion of measures of a proinflammatory state and oxidative stress (“Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III),” 2001) as several inflammatory markers have been identified that may provide additional clinical information about MetS and disease progression including CRP, MDA, and PGI_{2}, IL-6 and TNF-α (Di Lorenzo, Dell’Agli, Colombo, Sangiovanni, & Restani, 2013; Nielsen et al., 1997).
Hcy is a homologue of the amino acid cysteine formed by altered cystathionine-beta-synthase and methylenetetrahydrofolate reductase activity and associated deficiency of folic acid, vitamin B12 and vitamin B6. Oxidation of Hcy leads to the formation of homocysteine, homocysteine-mixed disulfides, and homocysteine thiolactone, which leads to the formation of superoxide anion radicals and hydrogen peroxide (Cipolla, Williamson, Nehler, Taylor, & Porter, 2000; Debreceni, 2001).

Elevated Hcy promotes atherosclerosis through increased oxidant stress, impaired endothelial function, and induction of thrombosis. Increases in plasma Hcy have been implicated in several disease processes including atherosclerosis affecting coronary, cerebral and peripheral arteries, chronic kidney disease (Sjoberg, Anderstam, Suliman, & Alvestrand, 2006) and Alzheimer’s disease (Cipolla et al., 2000; Clarke et al., 1998; Taylor et al.). Hcy-induced generation of reactive oxygen species contributes to vascular reactivity and pancreatic dysfunction leading to atherosclerosis and diabetes. The relationship between Hcy and coronary artery disease is especially pronounced in patients with diabetes.

A mechanism that has been associated with one or more of the five MetS factors, T2DM and CVD is oxidative stress and inflammation (Ridker, Stampfer, & Rifai, 2001; R. Ross, 1999; Skibinska et al., 2004). Oxidative stress and inflammation are linked through Hcy activity (Loscalzo, 1996; Oudi et al., 2010; Schalinske & Smazal, 2012). Homocysteine thiolactone reduces the generation of glycogen through oxidative stress mechanisms that has been confirmed by in vitro experiments that added GSH, a ubiquitous antioxidant, to Hcy induced oxidative stress in cell culture and reversed the reduction in insulin-stimulated glycogen synthesis (Najib & Sanchez-Margalet, 2001). Hcy and oxidative stress are linked by Hcy reducing glutathione peroxidase activity, which is important for the oxidation of GSH to GSSG (Bhabak & Mugesh, 2010). The relationship between 8-OHdG, GSH and its oxidised form GSSG has not been extensively investigated (Baez-Duarte et al., 2014; Schmitt, Vicenzi, Garrel, & Denis, 2015; Singhal, Nagaprashantha, Vatsyayan, Awasthi, & Singhal, 2011).

Hcy is associated with increased insulin resistance, increased blood pressure and increased LDL-cholesterol, which constitute three of the five MetS factors. It therefore becomes an important question which cluster of MetS factors are present in patients with metabolic syndrome and the association with Hcy and not whether MetS is present or not.

8-OHdG, a product of DNA base modification produced by the oxidation of deoxyguanosine, is considered as the most sensitive and useful marker of oxidative DNA damage (Nakajima et al., 2012; L. L. Wu, C.-C. Chiou, P.-Y. Chang, & J. T. Wu, 2004; T. Yano et al., 2009). GSH is a cellular antioxidant found in red blood cells. Levels of GSH were reported to be low in patients with T2DM, possibly due to impaired activity of the γ-glutamyl-cysteine synthetase enzyme (GCS), which is involved in the biosynthesis of glutathione, a lack of cysteine availability or damage.
to the erythrocyte membrane, which reduces the possibility of substances required in GSH production to cross the membrane (Tiwari, Pandey, Abidi, & Rizvi, 2013a; Yoshida et al., 1995).

Methods

A case-control study at a rural diabetes health (DiabHealth) screening clinic included 269 participants. The research was approved by the Ethics in Human Research Committee of Charles Sturt University. All participants received an information sheet and consented to the procedure. Participants for the study were attending the School of Community Health Diabetes Screening Clinic. NoMetS and MetS was defined according to the ATPIII definition. Self-reported medication use was also considered in classification of MetS in this research. Participants were comparable for age, gender, smoking habit, diet, and physical activity. Serum cholesterol levels and CRP was measured turbidimetrically at South West Pathology laboratories.

Measurements of oxidative stress. Erythrocyte MDA was measured using the thiobarbituric acid reacting substance (Stocks & Dormandy, 1971). Levels of MetHb were assessed using spectrophotometry of hemoglobin absorption before and after cyanide addition, and oxidative DNA damage was measured using the serum 8-OHdG ELISA Kit (Cayman Chemical, MI, USA). The kit utilises an anti-mouse IgG-coated plate and a tracer consisting of an 8-OHdG-enzyme conjugate. This format has the advantage of providing low variability and increased sensitivity compared to assays that utilize an antigen-coated plate. The level of erythrocyte reduced GSH was determined using the 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) reaction (E. Beutler, Duron, & Kelly, 1963).

Statistical analysis. The data was analysed using SPSS (Version 22) and Microsoft Excel (Office 2011, Microsoft). All values were expressed as mean ± standard deviation (M±SD). Statistical analysis was performed using a one-way ANOVA followed by LSD post-hoc test for between group comparisons. Pearson correlation, taking p < 0.05 as the significance limit was used to determine whether there was a significant correlation between Hcy and the other parameters associated with oxidative stress measured in this study.

Results

Traditional biochemical markers including WC, SBP, TC, TG, HDL, and LDL, blood glucose levels and HbA1c were all significantly different between no MetS and MetS groups. Of the emerging biomarkers Hcy, and 8-OHdG were significantly elevated in the MetS group compared to the no MetS group (Table 1).
Table 1. Traditional and emerging biomarkers for MetS syndrome

<table>
<thead>
<tr>
<th></th>
<th>No MetS</th>
<th>MetS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>66±11</td>
<td>67±9</td>
<td>ns</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>92±12</td>
<td>106±11</td>
<td>0.0001</td>
</tr>
<tr>
<td>BMI (cm/kg²)</td>
<td>26±4</td>
<td>31±5</td>
<td>0.0001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>129±18</td>
<td>135±15</td>
<td>0.003</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>76.5±9</td>
<td>77.8±11</td>
<td>ns</td>
</tr>
<tr>
<td>BGL (mmol/L)</td>
<td>4.9±0.6</td>
<td>6.5±2.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.7±0.4</td>
<td>6.3±1.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>5.2±1</td>
<td>4.9±1.4</td>
<td>0.023</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.4±0.3</td>
<td>1.2±0.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.3±0.9</td>
<td>2.8±1.2</td>
<td>0.001</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.1±0.5</td>
<td>1.9±0.9</td>
<td>0.0001</td>
</tr>
<tr>
<td>Hcy (μmol/L)</td>
<td>9.5±2</td>
<td>10.6±3</td>
<td>0.03</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>3.5±4</td>
<td>4.1±3.8</td>
<td>ns</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>31.7±27</td>
<td>41.1±45</td>
<td>ns</td>
</tr>
<tr>
<td>MDA (μmol/L)</td>
<td>13.9±7</td>
<td>16.2±8</td>
<td>ns</td>
</tr>
<tr>
<td>GSH (mg/100mL)</td>
<td>67.6±13</td>
<td>67.8±13</td>
<td>ns</td>
</tr>
<tr>
<td>8-OHdG (x10³ pg/ml)</td>
<td>307±516</td>
<td>1130±1155</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Demographic, anthropometric and clinical results were obtained from the cohort. Table 2 shows demographic and clinical results for the 5 factors of MetS.

Table 2. Demographic and clinical data associated with metabolic syndrome factors present

<table>
<thead>
<tr>
<th></th>
<th>0 MetS</th>
<th>1 MetS</th>
<th>2 MetS</th>
<th>3 MetS</th>
<th>4 MetS</th>
<th>5 MetS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>8/20</td>
<td>26/33</td>
<td>28/44</td>
<td>30/27</td>
<td>19/18</td>
<td>7/6</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>64.1±11</td>
<td>66.1±12</td>
<td>67.1±11</td>
<td>66.6±10</td>
<td>68.4±9</td>
<td>71.5±4</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>82.9±10</td>
<td>90.9±10</td>
<td>97.0±13</td>
<td>102.8±9</td>
<td>111.0±13</td>
<td>110.8±10</td>
</tr>
<tr>
<td>BMI (cm/kg²)</td>
<td>24±2</td>
<td>26.1±4</td>
<td>28.7±4</td>
<td>30.1±5</td>
<td>32.4±5</td>
<td>31.3±4</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>114±12</td>
<td>128.6±13</td>
<td>135.1±20</td>
<td>134.8±16</td>
<td>135.8±14</td>
<td>137.5±14</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>70.6±7</td>
<td>75.9±6</td>
<td>79.6±11</td>
<td>78.8±11</td>
<td>77.1±9</td>
<td>74±10</td>
</tr>
<tr>
<td>BGL (mmol/L)</td>
<td>4.7±0.6</td>
<td>5±0.5</td>
<td>5±0.7</td>
<td>6.2±2</td>
<td>6.4±3</td>
<td>8.6±4</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.6±0.3</td>
<td>5.7±0.4</td>
<td>5.7±0.4</td>
<td>6±0.6</td>
<td>6.5±2</td>
<td>7±2</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>5.3±0.9</td>
<td>5.4±1</td>
<td>5.1±1</td>
<td>5.1±1</td>
<td>4.7±1</td>
<td>4.4±1</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.6±0.2</td>
<td>1.4±0.3</td>
<td>1.4±0.3</td>
<td>1.3±0.3</td>
<td>1.1±0.2</td>
<td>1±0.1</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.4±0.8</td>
<td>3.5±0.9</td>
<td>3.2±0.9</td>
<td>3.1±1</td>
<td>2.8±1</td>
<td>1.9±1</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>0.8±0.3</td>
<td>1±0.4</td>
<td>1.2±0.5</td>
<td>1.6±0.8</td>
<td>2±0.8</td>
<td>3.1±0.9</td>
</tr>
<tr>
<td>BGL-lowering (%)</td>
<td>0</td>
<td>1.6</td>
<td>5.5</td>
<td>22.8</td>
<td>40.5</td>
<td>53.8</td>
</tr>
<tr>
<td>Chol-lowering (%)</td>
<td>0</td>
<td>8.4</td>
<td>22.2</td>
<td>31.5</td>
<td>62.1</td>
<td>61.5</td>
</tr>
<tr>
<td>BP-lowering (%)</td>
<td>0</td>
<td>22</td>
<td>45.8</td>
<td>61.4</td>
<td>83.7</td>
<td>76.9</td>
</tr>
</tbody>
</table>

All clinical markers increased with respect to the number of MetS factors present except LDL-cholesterol, most likely due to the increase in cholesterol lowering
medication use. HDL-cholesterol decreased in line with worsening pathology with increasing MetS factors present. Age was not significantly different between groups. WC was significantly different between all MetS factor groups (p<0.01), except for 4 MetS versus 5 MetS. In addition the group with no MetS factors present was significantly different from all groups with one to five MetS factors present (P=0.0001). DBP for the no MetS factors group was significantly different from the groups with one to four MetS factors present (p<0.05). Fasting BGL rose steadily and showed significant differences between no MetS factors and three to five MetS factors present (p=0.0001). Significant increases were also observed between 1 MetS and 3, 4 and 5 MetS factors present (p=0.0001); 2 MetS versus 3, 4 and 5 MetS (p=0.0001); 3 MetS and 5 MetS (p=0.0001) and between 4 MetS and 5 MetS (p=0.0001). Of the cholesterol markers, the triglycerides and HDL-cholesterol were most affected by addition of a metabolic risk factor, with TG’s rising significantly between groups, whilst HDL decreased (p<0.02). LDL-cholesterol also increased between 0 MetS versus 4 and 5 MetS (p<0.03); Between 1 MetS versus 3, 4 and 5 MetS (p<0.03) and between 2, 3 and 4 MetS versus 5 MetS (p<0.02).

Levels of inflammatory and oxidative stress markers as a function of the number of MetS factors present are shown in Table 3.

Table 3. Inflammatory and Oxidative stress markers in metabolic syndrome

<table>
<thead>
<tr>
<th></th>
<th>0 MetS</th>
<th>1 MetS</th>
<th>2 MetS</th>
<th>3 MetS</th>
<th>4 MetS</th>
<th>5 MetS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hcy (μmol/L)</td>
<td>8.6±3</td>
<td>9.5±3</td>
<td>9.7±3</td>
<td>10.9±3</td>
<td>10.6±3</td>
<td>9.4±3</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>2.1±3</td>
<td>4.1±6</td>
<td>3.7±2</td>
<td>4.7±5</td>
<td>3.1±2</td>
<td>3.8±3</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>29±20</td>
<td>38±30</td>
<td>24.4±29</td>
<td>129.4±261</td>
<td>46.3±29</td>
<td>30±27</td>
</tr>
<tr>
<td>MDA (μmol/L)</td>
<td>14.7±6</td>
<td>14.2±8</td>
<td>12.8±6</td>
<td>15.2±9</td>
<td>17.8±9</td>
<td>16.7±8</td>
</tr>
<tr>
<td>GSH (mg/100mL)</td>
<td>67±11</td>
<td>67.1±16</td>
<td>68.7±11</td>
<td>69.2±12</td>
<td>65±12</td>
<td>70.1±15</td>
</tr>
<tr>
<td>8-OHdG (x10^3 pg/ml)</td>
<td>0.2±0.16</td>
<td>0.2±0.14</td>
<td>0.5±0.8</td>
<td>0.9±0.1</td>
<td>1.2±1.1</td>
<td>1.7±1.4</td>
</tr>
</tbody>
</table>

Inflammation and oxidative stress increases with the number of MetS factors present in the cohort. The most dramatic increase was seen in 8-OHdG, which nearly doubled between 2 MetS and 3 MetS factors present (p<0.001). CRP rose significantly between 0 MetS versus 1 MetS and 3 MetS (p<0.05). MDA is a general oxidative stress maker and was significantly different between 2 MetS and 4 MetS only (p=0.01). In the current study, GSH showed a steady rise initially with increasing number of MetS factors present but no significant changes were noted.

Hcy levels steadily increased up to any 3 MetS factors present and then declined slightly (Figure 1). Significant differences for Hcy were found between no MetS factors present and 3 MetS (p=0.001) and 4 MetS factors (p=0.007) ; as well as between 1MetS and 3MetS (p=0.013) and 2MetS versus 3MetS (p=0.034). The decrease in Hcy levels observed between 4 and 5 MetS factors was not significant.
Figure 1 provides a comparison of the levels of the biomarkers Hcy, 8-OHdG and GSH relevant to the number of MetS factors present.

Discussion

An important caveat of the current study is that we applied the ATPIII definition of MetS to the study cohort and included glucose-lowering, blood pressure-lowering and cholesterol-lowering medication use as a further criterion for inclusion in the hyperglycemia, increased blood pressure and increased cholesterol factors of MetS. Our reasoning for this was that we concentrated on a population sample to determine changes in Hcy and whether these were significant and clinically usable as indicators of CVD and diabetes risk. Previous work suggests that 8-OHdG and GSH may be a useful clinical indicators. Endothelial function and insulin resistance was improved in patients with metabolic syndrome after folate and Vitamin B12 therapy (Setola et al., 2004). Homocysteine thiolactone also decreased in culture cells when GSH was added to the medium and improved insulin signalling (Najib & Sanchez-Margalet, 2001).

The definition of MetS does not consider factors such as age, gender, low density lipoprotein and emerging metabolic factors such as oxidative stress, inflammatory and coagulation markers (H.F. Jelinek et al., 2014; L. Maschirow et al., 2015). Endothelial dysfunction is a hallmark of CVD and diabetes suggesting that markers indicating endothelial dysfunction may be clinically relevant in assessing metabolic syndrome and risk of CVD and diabetes. Hcy is one such marker that is found in increased concentration in blood due to genetic or pathophysiological processes (Debreceni, 2001). By-products of Hcy degradation then play a role in oxidative stress (Ridker et al., 2001). 8-OHdG and GSH have been shown to be associated
with increased fasting BG levels, one of the factors in MetS (Al-Aubaidy & Jelinek, 2014; Hakki Kalkan & Suher, 2013; L. Maschirow et al., 2015).

Hcy may be a useful marker together with 8-OHdG in assessing the extent of metabolic syndrome and the impact of medication. Our results confirm previous findings that Hcy is not correlated with 8-OHdG (Kuwahara et al., 2013) nor with GSH. However GSH increased steadily up to four MetS factors when it decreased dramatically and then increased when five MetS factors were present. The decrease may be an artefact but may also be a result of downregulation by Hcy, which steadily increased with addition of MetS factors. GSH, which is oxidised to GSSG in the presence of hyperglycaemia may also have contributed the drop seen in GSH levels in addition to the antioxidant effects of medication use. We propose that GSH displays a cyclic change with disease progression, first increasing as oxidative stress increases but decreasing with increased oxidative stress prior to de novo synthesis, which leads to a secondary increase in GSH. This secondary increase in GSH is further enhanced due to the decrease in Hcy as seen in our study, which may be due to the increase in medication (Table 1) (Diane E. Handy, Yufeng Zhang, & Joseph Loscalzo, 2005).

Results were not adjusted for age, gender or creatinine as reported previously in a study comparing Hcy levels in people with MetS and vascular disease as our study concentrated on determining whether Hcy is a suitable marker for MetS in a population diabetes health (DiabHealth) screening initiative. We found significant results for Hcy associated with either MetS (≥3 factors present) versus noMetS (<3 factors present) in the cohort as well as a relationship between Hcy levels and an increase in the number of MetS factors included in the analysis, which was also reported previously (Hajer, van der Graaf, Olijhoek, Verhaar, & Visseren, 2007).

Whether Hcy is a causative factor in disease development or simply a biomarker is controversial with possibly links in diabetic nephropathy (Jia et al., 2015; H. Wang, Cui, Xu, & Xu, 2015), muscle malfunction (Veeranki & Tyagi, 2013), Alzheimer’s disease (Zhuo & Praticò, 2010). Our study indicates that the significant results associated with Hcy and the clustering of MetS factors may be the result of complex pathophysiological metabolic interactions with 8-OHdG and glutathione, suggesting that pathological vascular oxidative processes become more pronounced with the increase in MetS factors.

Acknowledgements

Bev de Jong is gratefully acknowledged for her contribution collecting the data in the diabetes health clinic and Simon McDonald from the Spatial Analysis Unit at CSU for providing statistical support. Roche Australia provided the glucose measuring sticks and glucometers.

CONFLICT OF INTEREST

THE AUTHOR(S) DECLARE(S) THAT THERE IS NO CONFLICT OF INTEREST REGARDING THE PUBLICATION OF THIS ARTICLE
References


Hajer, G. R., van der Graaf, Y., Olijhoek, J. K., Verhaar, M. C., & Visseren, F. L. (2007). Levels of homocysteine are increased in metabolic syndrome patients but are not associated with an increased cardiovascular risk, in contrast to patients without the metabolic syndrome. *Heart, 93*(2), 216-220. doi: 10.1136/hrt.2006.093971


Yano, T., Shoji, F., Baba, H., Koga, T., Shiraishi, T., Orita, H., & Kohno, H. (2009). Significance of the urinary 8-OHdG level as an oxidative stress marker...


Chapter 8

Paper 7: Low serum creatinine levels as risk factor of diabetes mellitus: prediabetes considerations


As I developed this thesis a search of the literature of emerging markers produced a paper by Harita et al. in Diabetes Care 2009 (see listing 1 in references of our publication given intra). The conclusion of Harita that low levels of creatinine as a predictor of diabetes risk required testing as this was not consistent with previous findings. As the Chief Medical Scientist of the South West Pathology Service, with responsibilities included general oversight and management of the clinical laboratories, research approvals and the maintaining of the laboratory information system (LIS). During the development of my PhD thesis it was apparent that we had an untapped source of existing data on traditional markers relevant to the studies of diabetes, impaired glucose tolerance and normal controls. The co-authors of this paper - Dr Nwose’s and Bwititi assisted in its development. Mr Cann, a trainee scientist, under my instruction and direction accessed the data base premised upon the criteria I had provided and he assisted in data acquisition and. Dr’s Nwose, Bwititi and myself conducted the data analysis, reviewed the literature and commenced the write up. Dr Nwose took the lead on my behalf, which included data filtration, as at that time I went through a number of family bereavements. All authors agreed on the final draft for submission.

Our proposal in this study linked the pathogenicity of diabetes and the prediabetic (subclinical) stage which would precede the diagnosis of the disease.
We tested the findings that lower levels of creatinine is a risk factor for diabetes; we them surmised that if this is the case we should include the prediabetic state to observe if similar findings occurred. We accessed our laboratory information system (LIS) and reviewed 1016 glucose tolerance tests which were eventually sorted into 3 categories, namely control, prediabetic and diabetic. Results which did not have creatinine levels were excluded. Glomerular filtration rate estimation (eGFR) was also included in the analysis as they are routinely used to provide evidence of renal failure based on a cut off of >60. We did make an assumption in this study that the confounding effect of diabetic nephropathy and/or retinopathy complications may be insignificant at the time of testing. As the participants were de-identified and the outcome of this study did not provide any clinical benefit to be offered, contact with patients did not occur. An ANOVA and students t-test of the 3 categories did not produce any significant differences in creatinine or eGFR. Our findings could not support the use of low creatinine results as a risk factor for diabetes. It is known that homocysteine (Hcy) levels are correlated with creatinine, and Hcy is not decreased in prediabetes, this would add further support to the expectation that creatinine levels would not be decreased. Our studies, whilst acknowledging increased patient numbers should be reviewed, did not support the hypothesis that low creatinine levels can be used as a predictor of diabetes risk.

Paper’s 8, 9 & 10 investigated emerging biomarkers and comorbidities.
Low serum creatinine levels as risk factor of diabetes mellitus: prediabetes considerations

EU Nwose\textsuperscript{1}, P Bwittiti\textsuperscript{2}, NG Cann\textsuperscript{3} and E Butkowski\textsuperscript{4}

\textsuperscript{1}South West Pathology Service\textsuperscript{1}, 590 Smollett Street, Albury and School of Biomedical Sciences\textsuperscript{4}, Charles Sturt University, NSW 2640 Australia

Summary
Objective: It has been reported that low serum creatinine level is a risk factor of diabetes. We hypothesize that should this be true, serum creatinine levels would be lower and more prevalent in prediabetes than in normal individuals.

Materials and methods: 1017 glucose tolerance tests performed at South West Pathology Service of the New South Wales Health, Australia, in 2008 were sorted into normal (control), prediabetes and diabetes based on diagnostic interpretation. All cases with creatinine results in the control (n=48), diabetes (n=18) and prediabetes (n=36) groups were selected.

Results: Mean levels of serum creatinine levels in the controls (80±32µmol/L), diabetes (82±26µmol/L) and prediabetes (82±23µmol/L) were not statistically significantly different. The prevalence of low levels of serum creatinine is less in prediabetes (11%) than in the control (23%).

Conclusion: Further studies using a larger number and adjusting for confounding factors is needed to ascertain the role of low serum creatinine level as a risk factor of diabetes.

Keywords: Prediabetes, risk factors, serum creatinine

Résumé
Il a été reporté que le faible taux de sérum créatinine est un facteur de risque du diabète. Nous posons l’hypothèse selon laquelle ce serait vrai, le taux de créatinine dans le sérum serait plus faible et plus prévalent dans les pré-diabètes que chez les individus normaux. En 2008, 1017 tests de tolérance en glucose ont été réalisés au service des soins pathologiques situé au sud ouest de Wales, en Australie. Les sujets étaient groupés en normale (contrôle), pré-diabètes et diabètes basés sur la décision de l’interprétation. Tous les cas ayant des résultats de la créatinine étaient sélectionnés chez le groupe de contrôle (n=48), diabétiques (n=18) et pré-diabétiques (n=36). Les taux moyens de créatinine dans le sérum chez les groupes étaient de (80±32µmol/L) de contrôle, (82±26µmol/L) chez les diabétiques et (82±23µmol/L) chez les pré-diabétiques n’étaient statistiquement et significativement différents. La prévalence de faibles taux de créatinine est moins chez les pré-diabètes (11%) que chez le groupe de contrôle (23%). Des études avancées en utilisant une population importante et ajustant les autres déterminants sont nécessaires pour clarifier le rôle de faible de créatinine en sérum comme un facteur de risque du diabète.

Introduction
Diabetes mellitus (DM) constitutes a major health problem worldwide and its pathogenesis follows a sequence of progression that includes prediabetes or subclinical diabetes stage preceding the diagnosis of the disease. With hyperglycaemia as a risk factor, blood glucose level is highest in DM and higher in prediabetes compared to healthy individuals. Following the report that low serum creatinine level is a risk factor of diabetes [1], we hypothesize that should this be true, serum creatinine levels would be lowest in DM and lower in prediabetes compared to normal individuals.

Research design and methods
1016 glucose tolerance tests’ results, of 2008, recorded in the laboratory information system of the South West Pathology Service, Albury were sorted into four categories on the basis of diagnostic interpretation [1]. Five hundred and eighty-nine (589) were normal and of these 48 had serum creatinine results [2]. Two hundred and forty-six (246) were reported as consistent with impaired fasting glucose or impaired glucose tolerance and of these 36 had serum creatinine results. The N=36 were newly diagnosed prediabetics and constituted the prediabetes group in this study [3]. One hundred and sixty-nine (169) results were consistent with gestational or type 2 diabetes and of these 18 has serum creatinine result. The N=18 were newly diagnosed diabetics and constituted the diabetes group in this study [4].
were not concluded for different reasons that made decisive interpretation impossible. The individuals with creatinine results in the three groups were selected for statistical analysis using analysis of variance (ANOVA) and student's t-test.

In this study, results of glucose tolerance test from our archived clinical pathology data was discretionally used as selection criteria to identify the otherwise de-identified subjects who were newly diagnosed of diabetes or prediabetes, as well as those who have laboratory evidence of normoglycaemia. Blood glucose level was part of information used in decisive interpretation of the result based on which sorting into groups has been done. Results of glomerular filtration rate estimate (GFR Est.) were determined to establish absence of laboratory evidence of renal failure, based on our laboratory's cut-off point of GFR Est. >60. However, it is assumed in the study that the confounding effect of diabetic nephropathy and/or retinopathy complications may be insignificant at this stage. As participants in this study were de-identified and the outcome of this study provides for no direct or immediate personal clinical benefit to be offered, contact with patients was not made.

Results
Comparison of creatinine levels in the controls (80±32 μmol/L), diabetes (82±26 μmol/L) and prediabetes (82±23 μmol/L) show no statistically significant difference between the groups (Table 1).

**Table 1: Characteristics and central values of groups**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Prediabetes</th>
<th>Diabetes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (0f/m)</td>
<td>48 (27/21)</td>
<td>36 (20/16)</td>
<td>18 (9/10)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>47±18</td>
<td>58±15</td>
<td>53±17</td>
</tr>
<tr>
<td>S. Cr. (μmol/L) Means±SD*</td>
<td>80±32</td>
<td>82±23</td>
<td>82±26</td>
</tr>
<tr>
<td>S. Cr. (μmol/L) Median</td>
<td>74</td>
<td>78</td>
<td>79</td>
</tr>
<tr>
<td>GFR Est. Mean ± SD*</td>
<td>78±15</td>
<td>73±15</td>
<td>76±15</td>
</tr>
<tr>
<td>GFR Est. Median</td>
<td>75</td>
<td>82</td>
<td>82</td>
</tr>
<tr>
<td>Prevalence of low S. Cr.*</td>
<td>23%</td>
<td>11%</td>
<td>28%</td>
</tr>
</tbody>
</table>

Key: Type 2 and gestational diabetes; *baseline value: <60 μmol/L; *No statistical difference between groups; GFR = glomerular filtration rate; S. Cr.* = serum creatinine.

Discussion
Insulin is the principal hormone that regulates transport of glucose from blood into cells, including muscles. Deficiency of insulin or the insensitivity of the cellular receptor components play a role in the pathogenesis of all forms of diabetes mellitus. Nevertheless, diabetes is not a single disease entity. Its aetiology involves a host of factors including other genetic predisposition, obesity, other endocrine secretions beside insulin and pregnancy [2-4].

It has been reported that low serum creatinine level is a risk factor for diabetes [1]. This is a new theory that demands attention. The implication of creatinine values as a biomarker in the pathogenesis of diabetes mellitus has only been associated with renal complications, where albumin-creatinine ratio is a biomarker [5]. The new theory, which is being tested in this study by evaluation of archived clinical pathology, implies that serum creatinine levels would be lower and probably more prevalent in people with subclinical diabetes vis-à-vis prediabetes when compared to apparently normal individuals. Therefore, we sought to compare the levels of serum creatinine and prevalence of hypocrreatinemia in newly diagnosed diabetes, prediabetes and healthy control groups.

We report observation of serum creatinine level that is not statistically significantly different between the apparently healthy control group and clinically diagnosed prediabetes group (see Table 1). This observation is in line with the notion that diabetes pathogenesis is unassociated with low serum creatinine levels, except in certain end-stage renal disease [6]. This report is also in agreement with a case report where elevated serum creatinine was observed in a patient who has prediabetes and kidney transplant [7].

The observed prevalence of low levels of serum creatinine is highest in the diabetes group (28%) compared to 11% and 23% observed in prediabetes and normal groups, respectively (Table 1).
Risk factors of DM

Given diabetic nephropathy and the position of prediabetes in the pathogenesis/progression of diabetes, we could infer that our observation of higher average of serum creatinine levels plus lower prevalence of hypocreatinaemia in the prediabetes group does not support the report that low level of serum creatinine is associated with diabetes. Our small sample size may be a limitation and this is duly acknowledged. Furthermore, we also report a prevalence of low levels of serum creatinine that is lower in the diabetes group than in the healthy control group. Therefore, it would be necessary to rigorously and scientifically debate this theory before making a decisive position statement.

Ordinarily, a low creatinine level in the absence of abnormal albumin level could impact on the albumin-creatinine ratio, glomerular filtration rate (GFR) and the clinical usefulness of the laboratory result. It is known that homocysteine level is correlated to creatinine level and the former is not lower in prediabetes than in the controls group [8]. What this report demonstrates is that serum creatinine levels is not lower in prediabetes compared to normoglycaemia. Therefore, this study is unable to provide support of a possibility of low serum creatinine level as a risk factor in diabetes. We surmise that further research is required to substantiate the speculation.

A few cautions need to be brought to fore in any further research. This includes but not limited to the following:

- Although serum creatinine levels are correlated with creatinine generation rate and correlates with muscle volume, study has shown that serum creatinine below a cut-off point was associated with gastrointestinal symptoms, pulmonary edema and uraemic encephalopathy [6].

- While serum creatinine is a cause of limitations in the clinical usefulness of GFR as a renal function test [9], albumin creatinine ratio has been reported to show a gradual increase with diabetes progression [5]. GFR did not show any statistical significant difference between groups in this pilot study (Table 1).

- There is inverse relationship between serum creatinine and mean kidney length, which means that serum creatinine levels will depend in part on the length of the person’s kidney [10].

Prediabetes has been termed America’s largest healthcare epidemic [11]. It is estimated to affect 16% adult Australians [12]. Serum creatinine is a very common laboratory test, but not currently in consideration in the diagnosis or screening of diabetes or prediabetes. The diagnosis and management of prediabetes and type 2 diabetes continues to require reassessment to ensure that goals are attained [13]. Affirmation of the speculation that this laboratory index may be useful – or otherwise – in diabetes prediction is imperative.

We have tried to search for reports on studies that followed up on the speculation of Harita et al.; to no avail, which implies that this report is the first to investigate the new theory. If the publication is meant to be acted upon by the readers, or translated for clinical practice, it is thinkable that follow-up research reports vis-a-vis scholarly debates will be imperative. Thus, the contribution of this article is not speculation of any new theory, but to stimulate debate on one that is speculated. It is recommendable to do further study using larger sample size as well as adjusting for kidney size and muscle volume to ascertain low serum creatinine level as a risk factor of diabetes.

Conclusion

Our report did not affirm that low serum creatinine level is a risk factor of diabetes. To our knowledge, there has been no study that followed up on the report, which means that our report is the first to investigate the new theory. In order not to appear skeptical, we indicate a direction or implication (prediabetes) that would benefit from the theory, subject to further debate or studies to affirm the speculation.

Acknowledgements

This work has been made possible by the approval and material support of the management of South West Pathology Service Albury, Australia.

References


Received: 15/12/09
Accepted: 17/01/11
Chapter 9

Paper 8: Oxidative stress and inflammation associated with decreased fibrinolysis as an early marker for peripheral vascular disease stratification

Jelinek, H.F., Khalaf, K., Robinson, C., McDonald, S. Butkowski, E, Al-Aubaidy, H.A. and Wild, T. Oxidative stress and inflammation associated with decreased fibrinolysis as an early marker for peripheral vascular disease stratification: A clinical study, Wound Medicine, 2015, 8, 24-3

In continuance of my PhD thesis this paper investigated whether inflammatory, oxidative changes and coagulation markers differed in low vs high ankle brachial pressure index (ABPI). My role in this study was (a) Initial development of the paper as we were investigating the role of oxidative stress (OS), inflammatory and coagulation markers and their association with comorbidities associated with T2DM. (b) Examining the current literature relevant to the content of this publication. (c) Contributing to the writing of the introduction and discussion, interpretation of results and providing input and review into the final draft in preparation for submission.

A significant proportion of diabetics suffer from hypertension and likewise hypertensive patients may demonstrate insulin resistance. The investigation of emerging biomarkers and their relationship to comorbidities associated with T2DM will provide insightful information into prediction and pathogenicity of the hyperglycaemic state.

It is acknowledged that a number of factors influence the formation of atherosclerosis, including coagulation, fibrinolysis, hyperglycaemia, hyperlipidemia and hyperinsulemia. In this study a number of emerging biomarkers were conducted in conjunction with degrees of PVD, assessed by
ABPI, recognising its limitation of false elevations due to arterial calcification. PVD is problematic in diabetic patients. Foot ischemia due to atherosclerosis and vascular calcification may have differing pathologies. Of importance to this study, inter alia, was the finding that D-dimer differentiated between low and high ABPI. Other findings utilising oxidative stress indicia and traditional markers such as lipids and glucose confirm the need for multi-parameter markers to aid in the understanding of the progression to CVD. Inflammatory markers were able to provide some insight and discrimination between low ABPI and high ABPI, however further discriminatory work utilising biomarkers should be assessed. Overall there may be an interaction occurring between inflammation and coagulation with atherosclerosis with resultant diverse pathology. Oxidative stress whilst not significant did show increasing levels of 8-OHdG in the high ABPI. Further validation of these results by exploring the emerging biomarkers may provide greater understanding of etiology and progression of PVD and other comorbidities associated with hyperglycaemia.

Paper 9 provided further investigation into oxidative stress and inflammatory markers of T2DM comorbidities.
Oxidative stress and inflammation associated with decreased fibrinolysis as an early marker for peripheral vascular disease stratification: A clinical study

H.F. Jelinek a,⁎, K. Khalaf b, C. Robinson a, S. McDonald a, E. Butkowski a, H.A. Al-Aubaidy d, T. Wild c

a School of Community Health, Charles Sturt University, Albury, Australia
b Department of Biomedical Engineering, Khalifa University of Science, Technology and Research, Abu Dhabi, United Arab Emirates
c Spatial Analysis Network, Charles Sturt University, Albury, Australia
d School of Medicine, University of Tasmania, Hobart, Australia
e Dessau Municipal Hospital, Dessau, Germany

ARTICLE INFO

Article history:
Received 29 September 2014
Accepted 9 March 2015
Available online 25 April 2015

Keywords:
Inflammation
Fibrinolysis
Oxidative stress
Ankle brachial pressure index
Peripheral arterial disease
Arterial stenosis

ABSTRACT

Background: Peripheral vascular disease may manifest as either arterial stenosis or calcification of the arterial wall. Arterial stenosis is characterized by a lowered ankle-brachial pressure index (ABPI), whereas calcified arteries lead to an increased ABPI. The current study investigates whether inflammatory and oxidative stress markers differ in low versus high ABPI groups suggesting different biochemical processes.

Methods: Eighty-seven patients attending the diabetes complications clinic agreed to the study. Participants were divided into a low (<1.07) and a high (>1.23) ABPI tertile groups and compared for traditional and emerging biomarker differences. A clinical history and demographic data were obtained. Emerging biomarkers included C-reactive protein, interleukin-6, 8-hydroxy deoxyguanosine, D-dimer, reduced glutathione and malondialdehyde. Data was analyzed using a Student t-test with significance set at 0.05. A multivariate normal linear regression model was applied to determine the contributions of traditional and emerging biomarkers to the classification of low and high ABPI.

Results: No differences were observed between the two groups for the traditional biomarkers but D-dimer (p < 0.001) and glutathione (p < 0.05) were significantly different between the groups. The best model for predicting ABPI class included the emerging biomarkers C-reactive protein, D-dimer and Interleukin-6 combined with BGL, HbA1c and SBP (p < 0.001) as compared to the best traditional biomarker model of HbA1c, atherogenic index of plasma, low-density lipoprotein and SBP (p < 0.05).

Discussion: A decreased inflammatory component with increased oxidative stress is more likely to lead to calcification of the arteries and a higher ABPI, despite normal BGL, HbA1c and cholesterol profile. Our study shows that emerging biomarkers provide a sound basis for differentiating pathophysiological processes associated with low and high ABPI. Traditional biomarkers showed a high accuracy for identification of low ABPI in our model but failed to do better than chance for identification of high ABPI. This suggests different pathophysiological pathways that include oxidative stress and inflammatory components. Clinically, validation of novel and more comprehensive risk factors for PVD sheds light on the etiology of the disease and might provide new treatment strategies.

© 2015 Elsevier GmbH. All rights reserved.

Introduction

Peripheral vascular disease (PVD) is a growing health problem and the risk is amplified by other cardiovascular risk factors, including diabetes mellitus (DM), low-grade inflammation, hypertension, atherosclerosis and lipid disorders [1]. In fact, the presence
of PVD may be the first indicator of atherosclerotic cardiovascular disease (CVD) and cerebrovascular disease (CBVD), even in asymptomatic people and is a reliable indicator of congestive heart failure and cerebrovascular accident [2,3].

Several metabolic factors influence atherosclerosis including coagulation, fibrinolysis, hyperglycemia, hyperlipidemia, and hyperinsulinaemia. Results of in vitro studies suggesting possible associations between hyperglycemia and lowered fibrinolysis have not been replicated in human studies where enhanced thrombosis, rather than lowered fibrinolysis, leads to the increased atherosclerosis and cardiovascular disease observed in diabetes [4,5]. Therefore consistent relationships between fibrinolysis, coagulation and cholesterol levels with peripheral vascular disease are still lacking [6]. Abnormal coagulation seems to be the main causative factor for thrombosis and atherosclerosis [7]. Clinical research has also shown ongoing thrombogenesis in the subclinical progression of PVD [8]. Blood constituents that play a role in thrombogenesis and fibrinolysis include fibrinogen, D-dimer, plasminogen activator inhibitor (PA-I) and tissue plasminogen activator (tPA). In particular, D-dimer has been found to be prognostic of PVD and fibrinogen concentration is correlated with the ABI [9,10].

Despite atherosclerosis or vessel thrombosis being a hypercoagulation disease, not all biomarkers associated with procoagulation follow the expected trend. One of these is D-dimer. D-dimer is a fibrin breakdown product and is expected to increase with an increase in coagulation or with pre-coagulation diseases such as diabetes and CVD [11]. An increase in D-dimer has indeed been reported in many studies [12-15]. However, Yano et al. suggested that, at least, in type 2 diabetes hyper-fibrinolysis is present as the thrombo-modulus-thrombin complex triggers thrombin-activating fibrinolysis inhibitor (TAFI) [17]. Our own work has indicated that in type 2 diabetes, levels of D-dimer may be associated with the endogenous antioxidant response mediated by glutathione [11,18]. In the case of peripheral vascular disease several studies have implicated an increase in fibrinogen, cross-linked fibrin degradation products and plasminogen activator inhibitor, as well as several emerging biomarkers including C-reactive protein, glutathione peroxidase and 8-hydroxy-2-deoxyguanosine (8-OHdG) in the progression of peripheral vascular disease [19-22].

Peripheral vascular disease is a highly prevalent form of cardiovascular disease particularly in the asymptomatic form [23,24] and may be diagnosed using the ankle-brachial pressure index (ABI). Values below 1.0, 0.9 or 0.8 have been reported to define the occurrence of PVD, with 0.5 indicating severe ischemia [25-30]. An ABI above 1.3 or 1.4 is taken to indicate arterial calcification [31,32]. Medial arterial calcification produces falsely elevated readings through incompressibility of the vessels [30].

ABI has also been shown to correlate with risk of cardiovascular disease based on the Framingham diagnostic criteria [33] and an increased all-cause mortality [10].

The literature clearly identifies a positive association between lower ABI and adverse cardiovascular events but is not as clear on the association with arterial calcification [34,35]. However, despite ABI being a robust clinical tool for determining PVD and a clear negative association between PVD and ABI, no study thus far has shown how minor changes in ABI correlate to levels of fibrinolysis and emerging biomarkers in PVD or arterial calcification [36,37].

Although traditional screening models have greatly improved cardiovascular risk prediction, studies have shown that a considerable number of future cardiovascular events occur in individuals with only one or zero risk factors present [38-40]. Interestingly, emerging laboratory parameters that hold promise as risk predictors for macrovascular clinical events include oxidative stress indices, (antioxidants and lipid peroxidation indicators) such as reduced glutathione and malondialdehyde [41-44], and macrovascular event indices (endothelial dysfunction, hypercoagulability and stasis respectively indicated by homocysteine (Hcy), 8-OHdG, D-dimer and blood viscosity) [11,38,45-47]. Nevertheless, different parameters reflect different pathophysiological pathways and do not necessarily apply to all disease processes [41]. The purpose of this study was to investigate whether various inflammatory and oxidative stress markers differ in low versus high ABI groups, suggesting the involvement of different biochemical processes in vascular pathology.

Methods

Participants of the Diabetes Screening Clinic at Charles Sturt University were recruited from the community through announcements in the local newspaper, radio and television [48]. The study was approved by the Charles Sturt University Human Ethics Committee and complies with the standards set out by the Helsinki agreement for human research. Two hundred people from the local community in Southern NSW and northern Victoria attended the diabetes complications screening clinic (DiSeCr) and of these 87 agreed to the study. All participants were informed of the aims of the research and procedures as well as any risk associated with data collection before signing the consent form.

Determination of ABI

The ABI was determined using an examination plinth with participants in a supine position. Participants were rested for a minimum of 5 min before SBP measurements were taken and the ABI determined. Examination rooms were all of a comfortable temperature, with minimal noise during ABI measurement. Using a pressure cuff, a sphygmomanometer and a Doppler ultrasound unit (Hadeco ES - 1000 SPI, Hayashi Denki Co., Kawasaki, Japan) with an 8 MHz probe, SBP measurements were obtained from each brachial artery and each posterior tibial artery. The ankle SBP reading was then divided by the highest brachial SBP reading to give a numerical value for the ABI [33,49]. Afterwards participants were divided into a lower tertile with a cut-off of 1.07 and higher tertile at a cut-off of 1.23, the two groups - low and high ABI - were compared for biomarker levels.

Measurement of body mass index and waist circumference

Weight was measured using standardized beam weight scales without footwear and with only light clothes on. Height was measured with the participants barefoot and standing with the feet together. BMI is defined as weight in kilogram per height in meters squared, and is independent of gender and age. Overweight is defined as a body mass index (BMI) of 25.0-29.9 kg/m² and obese is considered as BMI ≥30.0 kg/m² [19]. Waist circumference was measured with a standard measuring tape halfway between the lowest rib and the top of the iliac crest. Normal range was defined as a waist measurement of less than 94 cm for men or 80 cm for women [50].

Biomarker analysis

Following an overnight fast, whole blood specimens were collected into heparin, preservative free heparin and EDTA tubes for analysis depending on the specific biomarker. Plasma was separated within 1 h by centrifugation at 1000 x g for 10 min. Plasma from heparin-containing tubes was immediately used for lipid analysis. Plasma from preservative free heparin tubes was kept at −80 °C for D-dimer analysis. D-dimer determination was performed using the MiniQuant™ procedure (ImmunoDiagnostics). Malondialdehyde (MDA) was based on the protocol of thiobarbitalic acid reactive substances method. Mean MDA levels for
controls was taken as 15.6 μM with a range of 13–19 μM from previous work [51]. Oxidative stress was measured with glutathione. Fresh blood was kept on ice for not more than 1 h to measure GSH. The level of erythrocyte GSH was determined using the 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) reaction [52]. The local pathology laboratory determined the level of C-reactive protein (CRP), interleukin-6 (IL-6) as measures of inflammation using immunometric EIA kits and venous blood glucose levels. Reduced GSH and 8-OHdG are indicators of oxidative stress and also measured using ELISA.

National Cholesterol Education Program (ATP III) guidelines were met, for prediabetes fasting glucose > 5.6 mmol/L and male abdominal obesity by the use of >102 cm as cut-off point and female abdominal obesity of >88 cm [53–55]. Atherosclerosis Index of Plasma (AIP) was defined as the logarithm to the base 10 of the ratio of fasting plasma triglycerides to HDL-C measured in mmol/L. Dubina et al. classified the risk of atherogenicity depending on the level of AIP into: AIP < 0.11 – low risk; AIP between 0.11 and 0.21 intermediate risk; and AIP > 0.21 high risk [56–58]. The logarithm of the ratio log(TG/HDL-C) corrects for the lack of a normal distribution of the parameter in the population sample and correlates with the presence of smaller low-density lipoprotein-cholesterol (LDL-C) [58]. Fasting plasma total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) were measured by standard techniques. TC and TG were determined with a commercial enzymatic kit. HDL-C was determined by immunoinhibition assay. Low-density lipoprotein cholesterol (LDL-C) was calculated according to the Friedewald formula [59].

Statistics

Normal distribution was determined by the Coefficient of Kurtosis < 3 [60]. Therefore, data were log transformed to enable statistical analysis by Spearman’s rank correlation using PAWs (version 22 for Windows). Our emerging biomarkers, D-dimer, IL-6, GSH, AIP and CRP data were not normally distributed, a finding similar to previous observations [61,62] and was therefore log transformed. Intergroup differences were determined using the Student T-test with p set and 0.05.

Before proceeding with formal modeling, to limit the potential of multicollinearity, all traditional biomarkers and emerging biomarkers were compared using Spearman’s Rank Correlation (rs). Variables with strong correlations (rs > 0.7) were excluded from further analysis [63].

All combinations of all variables were modeled using logistic regression and ranked according to AICc. AICc is a second order variant of Akaike’s Information Criterion (AIC) and is corrected for finite sample sizes. AICc converges to AIC for large n and should be employed regardless for model selection [64]. Models that have a change in AICc of less than 2 have substantial empirical support [65]. The top models were checked for goodness of fit and significance measures (p value, pseudo R-squared and classification table) before selection.

Results

A total of 87 participants selected from 200 patients attending the diabetes screening clinic had full blood results. Of these, 43 had an ABIp value of less than 1.07 and 44 had ABIp values above 1.23. Table 1 shows the demographics of the two groups and indicates any significant differences observed using a Student T-test with p set at <0.05. Average ABIp values were significantly different between the two groups (p = 0.0091) as were the left and right ABIp (p = 0.0001) for the two groups (Table 2).

<table>
<thead>
<tr>
<th>Table 1: Group demographics.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feature</td>
</tr>
<tr>
<td>Age (yr)</td>
</tr>
<tr>
<td>Waist circumference</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
</tr>
</tbody>
</table>

Student T-test significant at p < 0.05.

BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure.

Traditional biomarkers were not statistically different between groups. Blood glucose levels (BGL) were within the prediabetic range in the low ABIp group but normal in the high ABIp group. The atherogenic index of plasma (AIP) is within normal limits but indicates a greater risk for atherosclerosis and cardiovascular disease in the low ABIp group. Total cholesterol (TC) is within a desirable range as are triglyceride (TG) levels in both groups. Low-density lipoprotein cholesterol (LDL-C) levels are elevated indicating an increased CVD risk especially in the low ABIp group. Only high-density lipoprotein cholesterol (HDL-C) is below the ideal value of 1.6 mmol/L for both groups (Table 3).

Table 4 shows biomarkers that have been considered more recently in the literature and associated with an increased risk of cardiovascular disease including peripheral vascular disease and diabetes. Only D-dimer and GSH show significant differences between the low and high ABIp groups.

Previous work of ours has shown an interaction between fibrinolysis and the level of glutathione. We therefore investigated possible models for classification of ABIp, including only classical biomarkers or inclusion of emerging biomarkers. The results are indicated in Tables 5 and 6.

The smaller AICc indicates better goodness of fit when taking into consideration the complexity of the model but does not indicate whether the model is actually a good or poor fit. The classification table and p value indicate ultimate goodness of fit.
Table 4
Emerging biomarkers in assessment of peripheral vascular disease.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Low ABI</th>
<th>High ABI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (mg/L)</td>
<td>3.83 ± 4.8</td>
<td>2.3 ± 2.24</td>
<td>0.007</td>
</tr>
<tr>
<td>Hcy (nmol/L)</td>
<td>11.58 ± 7.8</td>
<td>10.4 ± 3.2</td>
<td>0.32</td>
</tr>
<tr>
<td>MDA (µM)</td>
<td>16.23 ± 6.7</td>
<td>13.8 ± 6.6</td>
<td>0.08</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>50.24 ± 6.8</td>
<td>37.3 ± 3.73</td>
<td>0.02</td>
</tr>
<tr>
<td>GSH (mg/100 mL)</td>
<td>66.01 ± 13.3</td>
<td>64.7 ± 12.1</td>
<td>0.024</td>
</tr>
<tr>
<td>8-OHdG (ng/g)</td>
<td>608.9 ± 606.5</td>
<td>1352.7 ± 1597.2</td>
<td>0.48</td>
</tr>
<tr>
<td>D-dimer</td>
<td>470.8 ± 515.5</td>
<td>2804.1 ± 4712.2</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

* Significant at p < 0.05.

CRP = high sensitivity C-reactive protein, Hcy = homocysteine, MDA = malondialdehyde, IL-6 = interleukin-6, GSH = reduced glutathione, 8-OHdG = 8-hydroxy-2-deoxyguanosine.

and significance respectively. Five hundred and eleven possible models were evaluated and the six best models based on the criteria that the change in AICc from model to model did not exceed 2, are shown here. Model 1 performs best with an average accuracy of 68.3% (p < 0.05) biased toward correct classification of low ABI.

Table 6 presents the results of the six best models when including emerging biomarkers for oxidative stress and inflammation in the ABI group classification. Seven thousand one hundred and thirty two possible combinations were investigated.

AICc indicates a significant improvement in model classification accuracy from model 6 to model 1. The latter is the best model in our study with a total accuracy of 93.6 (p < 0.0005). The model includes BGL and Hba1C as its primary classification features and then in order of priority includes CRP, D-dimer and interleukin-6. Model 2 with Glutathione, CRP, D-dimer and interleukin-6 together with LDL and SBP as the main classification features is not significantly different to model 1 (p = 0.0004). This finding further indicates the importance of inflammation, oxidative stress and fibrinolysis in differentiating between pathophysiology associated with lowered and elevated ABI. Although there is a significant difference between the best and worst model when including emerging biomarkers in our study, model 6 still retains GSH and IL-6 as two important features for classification accuracy.

(= 0.0009). All models including the emerging biomarkers perform much better with AICc approximately halved when including the emerging biomarkers.

Discussion

Currently the ankle brachial pressure index is the standard measure used in clinical practice to identify the presence, and monitor the progression, of peripheral vascular disease. False elevated ABI readings associated with medial arterial calcification are however a limitation, as the score may underestimate the severity of vascular pathology. Reliance on ABI alone for the diagnosis of peripheral vascular disease is problematic, particularly in people with poorly controlled or long-standing diabetes [66].

Several biomarkers have been associated with PVD [67,68]. The current study identifies emerging biomarkers that vary significantly depending on whether lower or elevated range ABI values are investigated and suggest that the pathophysiology of atherosclerosis leading to foot ischemia is different to that observed with vascular calcification. The former emphasizes the importance of oxidative stress and inflammation related changes in the vasculature. Together with traditional biomarkers and possible formation of atherosclerosis. In contrast, arterial sclerosis is associated with significantly lower D-dimer levels and thus fibrinolysis, with no significant changes in inflammatory or oxidative stress markers apart from a non-significant increase in 8-OHdG, which is more indicative of vascular calcification. This finding corroborates previous results showing that an increase in 8-OHdG is associated with decreased flow mediated dilation and thus endothelial dysfunction [69]. Accepted pathophysiological processes associated with atherosclerosis suggest that vessel inflammatory processes lead to the recruitment of monocytes that turn into activated macrophages stimulated by oxidized-LDL. Macrophages ingest oxidized-LDL, enlarge and then eventually rupture [70]. As a continuum of this process macrophages also release calcium ions, which together with foam cells lead to the hardening of arteries [71]. However, sclerotic changes can be

Table 5
Classification of ABI groups with traditional biomarkers.

<table>
<thead>
<tr>
<th>Features</th>
<th>AICc*</th>
<th>p</th>
<th>Low ABI accuracy (%)</th>
<th>High ABI accuracy (%)</th>
<th>Average accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hba1c (AP)/LDL/SBP</td>
<td>67.2</td>
<td>0.01</td>
<td>83.1</td>
<td>54.5</td>
<td>68.3</td>
</tr>
<tr>
<td>2. Hba1c (AP)/LDL</td>
<td>67.3</td>
<td>0.013</td>
<td>83.7</td>
<td>45.5</td>
<td>65.6</td>
</tr>
<tr>
<td>3. BGL (AP)/SBP</td>
<td>67.6</td>
<td>0.006</td>
<td>80.0</td>
<td>50.0</td>
<td>60.9</td>
</tr>
<tr>
<td>4. BGL (Hba1c (AP)/SBP</td>
<td>67.2</td>
<td>0.01</td>
<td>77.8</td>
<td>45.5</td>
<td>61.4</td>
</tr>
<tr>
<td>5. AP/LDL/SBP</td>
<td>67.85</td>
<td>0.006</td>
<td>77.8</td>
<td>54.5</td>
<td>67.2</td>
</tr>
<tr>
<td>6. BGL /AP/LDL</td>
<td>67.8</td>
<td>0.006</td>
<td>80.0</td>
<td>54.5</td>
<td>67.2</td>
</tr>
</tbody>
</table>

* AICc = Akaike's Information Criteria.

Hba1c = glycated hemoglobin, AP = atherogenic index of plasma, LDL = low density lipoprotein cholesterol, SBP = systolic blood pressure, BGL = blood glucose level.

Table 6
Classification of ABI groups including emerging biomarkers.

<table>
<thead>
<tr>
<th>Features</th>
<th>AICc*</th>
<th>p</th>
<th>Grp1 accuracy (%)</th>
<th>Grp2 accuracy (%)</th>
<th>Average accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. BGL /Hba1c (AP)/LDL/SBP</td>
<td>27.4</td>
<td>0.0001</td>
<td>93.8</td>
<td>93.6</td>
<td>93.6</td>
</tr>
<tr>
<td>2. CRP /GSH (LDL)/LDL/SBP</td>
<td>28.1</td>
<td>0.0004</td>
<td>92.9</td>
<td>90.3</td>
<td>91.5</td>
</tr>
<tr>
<td>3. GSH (LDL)/AP/LDL/SBP</td>
<td>29.2</td>
<td>0.001</td>
<td>88</td>
<td>87.7</td>
<td>87</td>
</tr>
<tr>
<td>4. BGL(CRP)/(AP)/SBP</td>
<td>28.3</td>
<td>0.0004</td>
<td>88.9</td>
<td>80.2</td>
<td>87</td>
</tr>
<tr>
<td>5. BGL/Hba1c (AP)/LDL/AP</td>
<td>30.3</td>
<td>0.002</td>
<td>93.3</td>
<td>66.7</td>
<td>80</td>
</tr>
<tr>
<td>6. GSH (LDL)/AP/FGT/LDL/SBP</td>
<td>30.1</td>
<td>0.0009</td>
<td>94.1</td>
<td>85.7</td>
<td>90.9</td>
</tr>
</tbody>
</table>

* AICc = Akaike's Information Criteria.

Hba1c = glycated hemoglobin, AP = atherogenic index of plasma, LDL = low density lipoprotein cholesterol, SBP = systolic blood pressure, BGL = blood glucose level, CRP = high sensitivity C-reactive protein, DD = 6-dimer, IL-6 = interleukin-6, GSH = reduced glutathione.
independent of atherosclerosis and have been shown to be associated with renal disease [72]. Intimal calcification is associated with an endocrine imbalance manifesting as an increased level of phosphate, lower serum albumin and higher calcium carbonate intake and associated with advanced peripheral vascular disease [72]. Arterial calcification and vessel hardening have been reported to be more inflammatory, with cholesterol deposition, predominantly found in conduit arteries such as femoral, and tibial arteries [73].

Our results confirm that including emerging biomarkers, especially D-dimer differentiates between the low and high ABI groups. This suggests that the process of fibrinolysis is an important pathophysiological process that differentiates between PVD progression associated with arterial calcification and increased ABI. Oxidative stress, measured by GSH, is also included in two models together with the fibrinolysis marker, D-dimer, CRP and IL-6 being the inflammatory markers in the model.

Blood glucose levels, HbA1C and cholesterol profile combined with SBP had a high classification accuracy for lowered ABI, but not for the higher ABI group, suggesting that these markers play a more important role in pathophysiological processes leading to atherosclerosis, vascular stenosis and lower ABI. The best traditional biomarker was AIP, which has been shown previously to be highly associated with an increased risk of cardiovascular disease and also prediabetes [74,75].

Our current work extends findings relating traditional and emerging biomarkers in a multivariate paradigm, which provides better insight into increased risk of cardiovascular disease. The ABI cut-off values correspond closely to suggested cut-off values for PVD of <1.0 and arterial calcification of ABI > 1.2–1.3 [76–78]. The low ABI group had a mean BGL above the prediabetes cut-off, but lipid levels within the normal range. The slightly elevated but not significant increase in MDA in the high ABI group is less than the average level of MDA determined in a previous control study [51], suggesting that lipid peroxidation does not play a major role in increased ABI and the vascular calcification process, but is important in PVD when ABI values are lower due to oxidative stress reactions [70].

Oxidative stress measured as 8-OHdG and GSH levels in conjunction with traditional biomarkers such as cholesterol and BGL indicate that a multi-marker paradigm is essential to understand the progression of cardiovascular disease [43,80,81]. This is in agreement with discussions in the literature indicating the potential of emerging biomarkers. C-reactive protein (CRP) in the current study however, was not significantly different between the two groups, despite showing a much higher value in the low ABI group, which indicates that an inflammatory process is more likely found in the low ABI group than in the high ABI group [82,83]. In addition CRP is included in our classification models. The non-significant rise in CRP may be due to the small cohort studied here. However, previous work has shown that subclinical inflammation is correlated with peripheral vascular disease in a cohort where abnormal ABI was defined as less than 0.8 [84]. In our study we show that CRP is already slightly increased with a moderate decrease in ABI; the median normal concentration of CRP being 0.8 mg/L with a maximum cut-off at 3 mg/L, indicating that our low ABI group had on average higher CRP values and thus a higher risk of atherosclerosis and metabolic syndrome such as diabetes [85]. CRP is also correlated with AIP, which in our study is higher in the low ABI group [74]. Similar increases were found for interleukin-6, which was increased more in the low ABI group and found to be above normal limits possibly indicating a pro-atherogenic state [86].

Peripheral vascular disease may indicate a hypercoagulability state but previous work has shown mixed results when investigating correlations between biomarker levels such as D-dimer and ABI [16]. D-dimer has been shown to increase or decrease, especially in association with diabetes and disease progression, where D-dimer is inversely correlated with GSH [18,87]. Increased D-dimer and IL-6 led to the greatest decline in ABI in the Edinburgh Artery Study [22]. An interaction between inflammation and coagulation, which is associated with atherosclerotic, nevertheless leads to a divergence of pathophysiological processes as D-dimer loses association with ABI when IL-6 predominates, which in turn promotes CRP production and a lowered ABI and atherosclerosis [84,88,89]. This further supports the hypothesis that inflammation rather than coagulation is the predominant cause for atherosclerosis manifesting with a decrease in ABI and an interactive effect of D-dimer with IL-6 leads to a worsening of PVD [90]. Higher D-dimer and inflammatory markers have also been associated with a more rapid decline in functional capacity of people with PAD [91]. Finally, although 8-OHdG was not significantly different between the two groups, the higher ABI group manifested a doubling of 8OHdG.

Validation of novel risk factors for PVD may allow earlier detection, an improved understanding of the etiology and progression, and the development of new and better-targeted therapies. Early identification of risk factors is important to enable timely intervention to slow the development and progression of PVD in the population. Reducing the risk of advanced vascular disease and associated cardiovascular and cerebrovascular pathology is a vital public health initiative toward improving individual health status and reducing the rapidly increasing costs of public health service provision.

Conflict of interest

None declared.

References

Chapter 10

Paper 9: Association of cytokine activity and the Stroop cognitive function test


In preparation for this publication, Mr Fabrègue and Ms Voigt were conducting an internship at Charles Sturt University (CSU), on secondment from their respective universities located in France and Germany. I was responsible for their supervision and training of the experimental procedures, particularly the analysis of the biomarkers investigated in this study. This included providing them with direction and supervision in the generation and collation of the data in preparation for statistical analysis. Assistance for statistical analysis was provided by the spatial analysis unit at CSU. Ms de Jong assisted in the data collation and preparation. Mr Mouquot provided additional data from a previous internship and Associate Prof Stackowiak provided expert input. I assisted Mr Fabrègue in the initial setup and final compilation of the manuscript. All authors approved the composition of the final draft prior to submission.

In this study we built upon the investigation of OS and inflammatory biomarkers into comorbidities associated with T2DM. We investigated a number of emerging biomarkers and their association with cognitive decline, a recognised comorbidity of patients with T2DM. The cognitive assessment
by the Stroop battery of testing utilises the reading interference test (RIT) and the naming interference test (NIT). Interleukins, monocyte chemotactic protein-1 (MCP-1), C reactive protein (CRP) and insulin like growth factor-1 (IGF-1) were tested. Results obtained showed CRP was significant for RIT ($p = 0.022$); IL-1β ($p = 0.039$), MCP-1 ($p < 0.01$) were significant for NIT. IL-10 ($p < 0.01$, IL-6/IL-10 ($p < 0.03$) were significant in RIT and NIT. Significant differences between inflammatory markers dependent upon either RIT or NIT and indicate the need to be aware that cognitive assessment may have differing neuro-molecular associations. Our findings with MCP-1 are in disagreement with previous high Stroop test scores, whether this relates to Alzheimer Disease (AD) or alternate pathophysiology associated with non-AD is to be determined. We also analysed inflammatory marker ratios noting significance in IL-6/IL-10 in RIT and NIT. The results of this study indicate that the use of inflammatory markers may improve specificity when conducting cognitive decline testing. To our knowledge this is a first study undertaken to demonstrate a connection between inflammatory markers and behavioural data and further asserts the need to analyse comorbidities associated with hyperglycaemia, T2DM and CVD.
Association of cytokine activity and the Stroop cognitive function test

Fabrègue, F.1,2, Butkowski, E.2, Voigt, A.2,3, Mouquot, G.1,2, de Jong, B.2 Stackowiak, F.J.2,4 and Jelinek, H.F.2,5,*

1 Faculty of Sciences, University of Poitiers, Poitiers, France;
2 School of Community Health, Charles Sturt University, Albury, Australia.
3 Institute of Chemistry and Biochemistry, Free University of Berlin, Germany
4 SRH University of Applied Health Sciences, Gera, Germany;
5 Australian School of Advanced Medicine, Macquarie University, Sydney, Australia.

Abstract

The association between mild cognitive impairment (MCI) and inflammation is not conclusive. Determining any association depends on the assessment test for MCI. The Stroop battery consists of the reading (RIT) and naming (NIT) interference tests. The current study investigated whether the RIT or NIT assessment of MCI affected the association with inflammation.
Ninety-six participants undertook the Stroop testing battery. Serum Interleukin (IL-10, IL-6, IL1-β), Insulin-like Growth Factor-1 (IGF-1), C-Reactive Protein (CRP) and the Monocyte Chemotactic Protein-1 (MCP-1) levels were measured using commercial ELISA kits.

CRP was significant for the RIT (p= 0.022). IL-1 β was associated with NIT (p= 0.039). MCP-1 was significant only for NIT (p<0.01), whereas the IL-10 (p<0.01) and IL-6/IL-10 ratio was significant for both RIT and NIT (p< 0.03).

The association between inflammatory markers and the Stroop results differ depending on whether the RIT or NIT was used. This suggests that both RIT and NIT should be used for clinical applications when inferring MCI and any association with inflammation.

Keywords

Inflammation, mild cognitive impairment, Stroop test, reading interference test, naming interference test.

Abbreviations:

IL – Interleukin

IGF-1 - Insulin-like Growth Factor-1

CRP - C-Reactive Protein

MCP-1 - Monocyte Chemotactic Protein-1

NIT – Naming Interference Test

RIT - Reading Interference Test
Introduction

Many factors such as cerebrovascular accidents, hypothyroidism, heart failure, head trauma, vitamin deficiencies, hepatic impairment, depression, drug and alcohol abuse influence cognitive function as well as toxins, infections, metabolic and structural causes. Furthermore MCI is also linked to obesity, vascular disease, dyslipidaemia, inflammation and oxidative stress (Darby, 2012; N. Lorius, J. J. Locascio, D. M. Rentz, K. A. Johnson, R. A. Sperling, A. Viswanathan, and G. A. Marshall, 2015; M. W. Marlatt, Paul J. Lucassen, George Perry, Mark A. Smith, and Xiongwei Zhu, 2008; J. C. D. Nguyen, S. Killcross, & T. A. Jenkins, 2014), pathologies which are all on the rise world-wide. Mild cognitive impairment (MCI) is a common age related phenomenon which may be a pre-clinical stage of dementia in general and Alzheimer’s Disease (AD) specifically. Episodic memory is most often affected by cognitive impairment, which can lead to AD dementia (Albert, 2011). How MCI is identified and whether it is affected by dyslipidaemia, inflammation and oxidative stress is important in clinical practice as it can determine treatment priorities.

Oxidative stress and inflammation associated with vascular disease have been proposed to be early markers of cognitive decline. One study suggested that the level of oxidative markers is directly related to the severity of cognitive impairment (Verri, 2012). Previous research has also shown a possible link between oxidative stress and cytokine production (A. A. Elmarakby, and Sullivan, J.C., 2012). Expression of inflammatory cytokines in neuronal cells suggests that inflammation and the production of ROS are closely related in the progression of cognitive decline (Madeo, 2013). Cholesterol has also been implicated with a causative role in MCI (Michikawa, 2003). However controversy still exists whether inflammation and MCI are related (Schram et al., 2007).
Cytokines are inflammation biomarkers, which play a role in enhancing or preventing disease progression. An imbalance of pro and anti-inflammatory cytokines, which may be caused by dyslipidaemia and leading to inflammatory cascades is related to changes in the levels of interleukin-6 (IL-6) and increases in other cytokines such as interleukin-1β (IL1-β) production (Fearon, 2008). The pro-inflammatory IL1-β is a crucial factor contributing to MCI and is a possible tool to predict neurodegenerative diseases (Forlenza, 2009).

Much of the current published research on MCI investigated only one biomarker and reported associations with either the Stroop RIT or NIT scores but did not apply both Stroop tests. C-reactive protein (CRP) (Laurin, 2009), Interleukin-12 (IL-12) (Rentzos, 2006), Interleukin-1 (IL-1) (Yucesoy, 2006), tumour necrosis factor-α (TNF-α) (Diniz, 2010) and monocyte chemotactic protein-1 (MCP-1) (D. Galimberti, Fenoglio, C., Lovati, C., Venturelli, E., Guidi, I., Corra, B. and Scalabrini, D., 2006) have been shown to significantly increase or decrease in MCI.

The link between pro-inflammatory and anti-inflammatory biomarkers and cognitive decline has only been shown in one study of patients with early or late onset Alzheimer’s disease (LOAD), (Dursun, 2015), reporting a significant higher serum IL-6 level in the LOAD group and a significant correlation with high serum IL-10 levels.

Cognitive tests are commonly used to determine the presence of MCI. Scores on cognitive tests for individuals with MCI are typically 1 to 1.5 standard deviations below the mean for their age and education matched peers on culturally appropriate normative data (Albert, 2011). Examples of such test batteries include the Differential Aptitude Test Battery, Cambridge Neuropsychological Test Automated Battery (CANTAB®) (Égerhazi, 2007) and the Vienna Cognitive Test Battery (Pusswald, 2013).
The Vienna Cognitive Test Battery includes a number of tests for attention, memory and visuospatial skills of which the colour-word interference tendency, (Stroop) test has been extensively investigated and often used to find a correlation between MCI and vascular disease (Prins, 2005). The principle of the STROOP test (Stroop, 1935) is that it is harder for an individual, to name the colour of the writing in which a different colour-word is shown on the computer screen, than to name the colour of the writing in which the same colour-word is displayed. Test results are interpreted by the median reaction time (seconds) and/or the number of incorrect responses made in relation to congruent and incongruent words. The interference between congruent and incongruent words can then be a predictor of MCI as interference deficits may be present before cognitive decline symptoms reach clinical levels (Bélanger, 2010).

Results of previous research investigating the relationship between MCI and inflammation are not conclusive and may be nonspecific (Schram et al., 2007; Yaffe et al., 2004). The role of anti-inflammatory biomarkers and their interaction with pro-inflammatory cytokines has not been investigated in the context of whether the Stroop RIT and NIT results are used with respect to inflammatory markers (O’Garra, 2012; C. J. Wilson, Finch, C.E., Cohen, H.J.. 2002). The current study aimed at investigating the association of pro- and anti-inflammatory biomarkers using both RIT and NIT.

1 Methods

The study was approved by the Ethics in the Human Research Committee of Charles Sturt University (Ethics approval number: 2006/042). Informed consent was obtained from each participant after providing a comprehensive outline of the project and methods followed by answering any questions participants may have had.
2.1 Study population

For the present study, 132 participants were recruited attending a rural health screening clinic for cognitive function testing using the Stroop test battery and analysis of blood biomarkers. We divided the results into high and low Stroop score for both RIT and NIT. Twenty-nine patients were excluded because blood samples were not available, while seven others did not complete the cognitive tests. The final number of participants for the study was 96. The participants were divided into two groups based on the normal range for the Stroop interference test obtained from 25 patients attending the health screening clinic who reported no hypertension (HT), cardiovascular disease (CVD), diabetes, psychiatric disease or cognitive difficulty. The cut-off values for the reading interference test (RIT) and naming interference test (NIT) were determined by setting the T-score value of the Stroop test results for RIT and NIT equal to 60, which represents one standard deviation from the normalised mean of the group. This provided a cut-off for RIT of > 240 milliseconds (msec), and for NIT of > 680 msec. Scores below the cut-off were deemed as normal cognitive function and represent the 0-85th centile for the current cohort. Comorbidities presenting in patients such as diabetes were defined as fasting plasma glucose levels (FPG) ≥126 mg/dL (7 mmol/l) (American Diabetes, 2012). Hypertension was defined as a systolic blood pressure (SBP > 120mmHg or self-reported hypertension with or without medication use. CVD was identified by 12-lead ECG, self-reported clinical history and use of medication.

2.2 Chemicals

The blood samples were analysed using ELISA kits (Elisakit.com, Melbourne, Australia) (Interleukin-6 (human) Elisa kit Lot No. 120925, Interleukin-10 (human) Elisa kit No. 13426, Interleukin-1β. (human) Elisa kit Lot No. 141208, Insulin-like growth factor-1 (IGF-1) (human) Elisa kit Lot No. 130807, and Monocyte
chemotactic protein (MCP-1) Elisa kit Lot No. 150413.

2.3 Sample preparation

Fasting plasma glucose (FPG) levels were determined using the Accu-Chek® system (Roche Australia Pty Ltd). 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 were carried out with a Thermo Scientific Multiskan FC (Fisher, China) (L. Maschirow, Khalaf, K., Al-Aubaidy, H.A., and Jelinek, H.F.. 2015). Venous blood was collected into serum-separating-tubes (SST) and stored at -80°C until analysis. All samples and standards were measured in duplicates. Blank and seven standards were used for each Elisa assay. The sample concentrations were calculated according to elisaanalysis.com from the optical densities (Thermo Scientific Multiskan FC).

2.4 Vienna Cognitive Test Battery

All participants completed the Stroop test included with the Vienna Cognitive Test Battery. Two baseline conditions and two interference conditions composed the Stroop test. First, a colour-word is displayed in grey and the patients had to read and then choose the correct colour field with a light-pen (for example, the colour-word "blue" written in grey, the answer is the blue-colour). In a second case, the patients have to choose the correct colour of a colour-line by saying the colour of the banner aloud (if the banner is "red", the answer is the red colour). Third, for reading-colour interference, the patients have to read the colour word aloud and disregard the colour the word is written in. (for example, the colour-word "yellow" written in green, the patients have to read aloud yellow and choose the yellow colour. The last test addresses naming-word interference. The patients have to name aloud the colour, in which the word is written and choose this colour (for example, the colour-word "red" written in green, the patients have to say aloud "green" and choose the green-colour.
The Stroop test assesses cognitive functioning by testing the underlying cognitive processes that are necessary to complete the Stroop RIT and NIT tasks effectively. Relevant and irrelevant information are processed in parallel. As the processing speed for reading a word is faster than naming a colour and more strongly automatized, it interferes with the task of naming the colour, when the colour stimulus presented on the screen and the colour in which the colour word is written are different.

2.5 Statistical analysis
Data were collected using Microsoft Excel (Office2007, Microsoft) and descriptive data is expressed as mean ± standard deviation (x ± SD). Statistical analysis was performed with SPSS (Version 22, IBM Co). To determine if there were significant differences in biomarker levels between the control and mild cognitive impairment, two samples Wilcoxon Rank Sum Test was used. A p-value of p ≤ 0.05 was considered as significant.

1. Results
Anthropometric data for the 96 adults who completed the cognitive function tests and for whom blood samples were available is shown in Table 1. All participants were older than 40 years. Sixty-two per cent of the study participants in the low Stroop group were women versus 35.7% in the high Stroop group. The mean age of the low Stroop group was 67 years and not significantly different to the high Stroop group. Only CVD and HDL-Cholesterol were significantly different between the high and the low Stroop group (p < 0.05).
Table 1. The demographics of high Stroop patients and low Stroop patients.

<table>
<thead>
<tr>
<th></th>
<th>Low Stroop</th>
<th>High Stroop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (n)</td>
<td>82</td>
<td>14</td>
</tr>
<tr>
<td>Gender (women) %</td>
<td>62.2</td>
<td>35.7</td>
</tr>
<tr>
<td>Age (years)</td>
<td>66.98 ± 9.5</td>
<td>69.71 ± 10.7</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>6.86 ± 2.8</td>
<td>6.81 ± 1.6</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>28.55 ± 5.8</td>
<td>30.57 ± 5.3</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>97.25 ± 14.8</td>
<td>102.89 ± 9.8</td>
</tr>
<tr>
<td>CVD (%)</td>
<td>22.78</td>
<td>53.85*</td>
</tr>
<tr>
<td>HT (%)</td>
<td>54.32</td>
<td>78.57</td>
</tr>
<tr>
<td>Type 2 Diabetes (%)</td>
<td>50.65</td>
<td>50</td>
</tr>
<tr>
<td>Tchol (mmol/L)</td>
<td>5 ± 1.1</td>
<td>4.49 ± 1.3</td>
</tr>
<tr>
<td>LDL-Chol (mmol/L)</td>
<td>2.85 ± 1.1</td>
<td>2.54 ± 1.2</td>
</tr>
<tr>
<td>HDL-Chol (mmol/L)</td>
<td>1.55 ± 0.5</td>
<td>1.26 ± 0.3*</td>
</tr>
<tr>
<td>HbA1c (mmol/L)</td>
<td>6.17 ± 0.9</td>
<td>6.17 ± 0.7</td>
</tr>
<tr>
<td>Tchol/HDL ratio</td>
<td>3.48 ± 1.3</td>
<td>3.71 ± 1.2</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.25 ± 1.5</td>
<td>1.51 ± 0.7</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>131.29 ± 17.9</td>
<td>130.86 ± 13.4</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>76.01 ± 7.6</td>
<td>76.14 ± 8.1</td>
</tr>
</tbody>
</table>

Abbreviations. HT: Hypertension; CVD: Cardiovascular disease. TChol: total cholesterol; LDL-Chol: low-density lipoprotein cholesterol; HDL-chol: high-density lipoprotein cholesterol; HbA1c: glycated haemoglobin; TG: triglycerides; SBP: systolic blood pressure; DBP: Diastolic blood pressure.

*: p < 0.05

Cut-off values for normal Stroop test results are also provided by Schuhfried, who developed the Vienna Test Battery but the Vienna cohort had an average age of 42
years (range 41-67) and Stroop results of 280 ms for RIT and at 240 ms for NIT. Due to the age difference between the Vienna cohort and our study, we determined our own cut-off values from a control cohort with appropriate age range. For the RIT our cut-off was slightly lower (RIT = 240 ms) but the cut-off for NIT was higher (668 ms) compared to the Schuhfried cut-off. Low and high Stroop group membership was based on either the RIT or NIT results.

Cytokines levels were determined depending on the RIT and NIT test results. For RIT, serum CRP levels were significantly higher in the high Stroop group compared with the low Stroop group (increase of 50%, p = 0.022) (Figure 1) and IL-10 was significantly lower with a decrease of 16.7% (p = 0.0096).

Figure 1. Impact of reading interference test score on normalised cytokine levels.

Values shown in the bar plots are the normalised means ± SD of all inflammatory biomarkers. The white bar plots represents the participants, who have a RIT value
below the cut-off (< 240 msec). The grey bar plots represents the participants, who have a RIT value above the cut-off (> 240 ms).

MCP-1 was significantly lower in the high Stroop group when the NIT classification was applied, (7.8%, p = 0.003). A decrease in MCP-1 levels was also noted when RIT was applied but was not statistically significant (decrease of 21.5% with p = > 0.05). IL-10 was also significantly lower in the high Stroop group (decrease of 6.3% with p = 0.003) but to a lesser extent than found when RIT was applied. Similarly CRP was lower by 44.5% in the high Stroop group but the result was not significant (Figure 2). The high Stroop group demonstrated a significant increase in IL-1β levels of 49% (p = 0.039) but IL-1β levels were not statistically significant on the basis of RIT (increase of 36% compare Figure 1 and Figure 2). IL-6 was elevated in the high Stroop group by 65% for the NIT but also not significant.

Figure 2. Impact of naming interference test score on normalised cytokine levels.

Abbreviations. CRP: C reactive Protein; IGF-1: Insulin-like growth-factor 1; IL-1β: Interleukin-1β; IL-6: Interleukin-6; IL-10: Interleukin-10; MCP-1: Monocyte chemotactic protein-1; NIT: Naming Interference Test. #: p < 0.06; *: p < 0.05; ***: p < 0.01.

Values shown in bar plots are the normalised means ± SD of all inflammatory
biomarkers. The white bar plots represents the patients, who have a NIT value below the cut-off (< 668 msec). The grey bar plots represents the patients, who have a NIT value above the cut-off (> 668 ms).

Only IL-10 levels decreased significantly regardless of the Stroop test applied (RIT: p = 0.0096 and NIT: p = 0.003).

The interactions between pro- and anti-inflammatory markers are an important component of disease progression. To obtain an understanding of this balance we used the ratio between IL-6, a pro-inflammatory cytokine and IL-10, an anti-inflammatory cytokine. Figure 3 indicates an increase in the IL-6/IL-10 ratio when applying RIT of 8% (p = 0.016) and an increase of 40% for NIT (p = 0.019).

Figure 3. Impact of reading-naming interference tests score on normalised cytokine IL-6/IL-10 ratio.

Abbreviations. IL-6: Interleukin-6; IL-10: Interleukin-10; RIT: Reading Interference Test; NIT: Naming Interference Test. **: p < 0.03.

Values shown in bar plots are the normalised means ± SD of the ratio IL-6/IL-10.

Discussion

Cognitive decline due to vascular impairment without dementia as a disease entity
has gained increased recognition in clinical practice. MCI can be assessed using the Stroop test where reaction time increases (high Stroop score) indicate possibly cognitive decline whether the RIT or NIT are used in clinical practice. However the current research results indicate that whether cytokines are significantly associated with the Stroop test battery depends on whether the RIT or NIT is applied. Here we used the RIT and the NIT to categorise participants into a high and low Stroop score groups and investigated how or if pro and anti-inflammatory cytokines were associated with the Stroop score.

An elevated Stroop score and therefore possible MCI was linked with a higher mean CRP level and a lower IL-10 level for RIT. Higher NIT scores were significantly associated with lower MCP-1 and IL-10 and higher IL-1β levels. Previous studies demonstrated higher level of IL-1β in serum samples of MCI patients (Forlenza, 2009), and others in AD patients (Angelopoulos, 2008; Khemka, 2014) with the AD group having a mean age similar to our study. Similarly, Koziorowski et al. detected higher IL-1β levels but no statistically significant change was demonstrated in Parkinson’s disease patients (Koziorowski, 2012). We found a significant increase in IL-1β only for NIT. The difference in inflammatory levels depending on the Stroop NIT and RIT performed with respect to IL-1β highlights the need to be aware that cognitive function tests may have different neuro-molecular associations even within the Stroop battery of tests.

Mean levels of the anti-inflammatory marker MCP-1 were significantly lower for NIT. Our results are in disagreement with some previous studies that have shown MCP-1 to be increased in MCI (high Stroop test scores) patients (D. Galimberti, Fenoglio, C., Lovati, C., Venturelli, E., Guidi, I., Corra, B. and Scalabrini, D.. 2006). However Galimberti and al. demonstrated that MCP-1 elevation is a very early event in AD pathogenesis and decreases with AD progression. The findings of Galimberti
et al. suggest that NIT may be a sensitive test for early cognitive decline but as our study showed a significantly lower MCP-1 level, AD pathogenesis may be different to the pathophysiology associated with non-AD related cognitive decline.

We also studied the association of the anti-inflammatory IL-10, with both the RIT and NIT scores. IL-10 acts to diminish pro-inflammatory cytokines (K. W. Moore, de Waal Malefyt, R., Coffman R.L., and O'Garra, A.. 2001). What the role of IL-10 is in MCI pathology is not clear. Previous results have shown both an increase and a decrease in IL-10 in association with Parkinson’s disease and multiple sclerosis (Patanella, 2009). Experiments with lipopolysaccharide (LPS) activated macrophages increased the level of IL-10 in AD patients (Lombardi, 1999). In our study, participants with known Parkinson’s disease and multiple sclerosis were excluded and thus the significantly lower IL-10 levels may be due to IL-10 acting as an anti-inflammatory cytokine in MCI as identified with Stroop.

We found no significant increase in IGF-1 in the high RIT or NIT score group but according to the literature, IGF-1 is a protecting factor against MCI suggesting that in our study IGF-1 may be on the rise or already decreased due to its protective function (Sonntag, Ramsey, & Carter, 2005). A future study investigating progression of MCI in our study group may be able to clarify whether IGF-1 increases as MCI progresses when classified by increased reaction times in the RIT and NIT results. IGF-1 was shown to be increased in association with the NIT in a cohort of 61 MCI patients, suggesting that IGF- is part of a complex response pattern (Baker, 2012).

No significant increase was observed for IL-6 associated with the RIT but we could see an increasing trend. For NIT, IL-6 was close to being significant (p= 0.052) supporting an increased IL-6 level reported previously in MCI patients (Dursun, 2015; Gorska-Ciebiada, 2015; Kalman J., 1997). Further, (Dursun, 2015), was the
first to show a significant relationship between IL-6 and IL-10 where high IL-6 levels led to an increased IL-10 level in an Alzheimer’s disease study. Our study consolidates this finding and shows that a similar pathophysiological relationship between IL-6 and IL-10 is found in MCI. In our study the high score RIT subgroup demonstrated an elevation in the IL-6/IL-10 ratio, suggesting that anti and pro-inflammatory biomarkers can play a role in the inflammatory response in MCI.

CRP is one of the most common inflammatory cytokines studied in pathological processes. CRP was increased in MCI (Metti, 2014), AD, T2DM (Pradhan, 2001). But there are two studies with no correlation between CRP levels and cognitive decline (Dik, 2005; Weuve, 2006). We found a significant increase in CRP when the high Stroop score was based on the RIT. According to the literature, NIT and RIT could be used together and can act in parallel to determine cognitive responses (Lindsay, 1991). However the majority of studies found in the literature used only the NIT. We are the first study to use the RIT and NIT and compare the test results with inflammatory biomarkers. We found that there were slight differences between the two tests. Some biomarkers were statistically different between the low and high Stroop score groups using one Stroop test but not statistically different when using the other Stroop test. Only IL-10 and the ratio IL-6/IL-10 were significantly different for both tests. These results are more reliable as the use of two simultaneous tests can improve the specificity and decrease the sensitivity if we consider that a patient is classified as positive when both tests are positive and negative otherwise. Reliance on accurate results that reflect the clinical profile of the patient is necessary to provide timely and effective treatment.

**Conclusion**

Our study demonstrated that the NIT and RIT Stroop tests are not correlated with the same inflammatory markers tested. Only IL-10 and IL-6/IL-10 were consistent
between the two Stroop tests in a population of patients attending our health screening clinic. It is the first study to demonstrate a connection between basic inflammatory biomarkers and behavioural data. Early cognitive neuropsychological testing and intervention can benefit patients, but the type of Stroop test used needs to be considered if neuroinflammatory processes are to be considered and appropriate treatment such as NSAID use initiated.

Acknowledgements
The authors wish to acknowledge the technical assistance by Bev de Jong and Roche Australia for providing blood glucose test strips and Glucose Reader. Simon McDonald from Spatial Data Analysis Network (SPAN) provided statistical support.

Statement of Interest: None to declare

References
Chapter 11

Paper 10: Acute Phase Inflammatory Response to Single-Bout HIIT and Endurance Training: A Comparative Study


Mr Kasper, the 1st author of this manuscript was an intern student from Germany. I conducted his technical training and supervised the analytical testing procedures (ELISA) of the cytokines, data collation and reduction. Dr’s Jelinek (principal researcher and supervisor) and Al-Aubaidy and I assisted Mr Kasper with the development of the paper and provided assistance with the literature search and the compilation. Ms. de Jong provided assistance with data collation, presentation and statistical analysis with Mr McDonald from the CSU Spatial Analysis Unit. Mr Perkins provided exercise data as part of his honours research with Dr Jelinek. All authors approved the manuscript prior to submission for publication.

The study implemented in this paper was to compare the biomarkers IL-1β, IL-6, IL-10, MCP-1, IGF-1 and CRP in clients undergoing acute single bout endurance testing (ET) and high-intensity interval training (HIIT). Exercise and physical activity are associated with a healthier lifestyle, lower levels of pro-inflammatory cytokines and higher levels of anti-inflammatory cytokines. Exercise is known to improve comorbidities in T2DM and in this study we have confirmed significance in certain inflammatory cytokines but
also the type of exercise conducted. Significant results were obtained with IL-6/IL-10 ratio (p = 0.047), and a decrease of MCP-1 (p = 0.03). Reduction in MCP-1 levels were significant in HIIT noting this form of exercise provides added cardiometabolic benefits. One finding would be that HIIT may present a valid alternative to ET, which has implications on obesity derived from inactivity on populations where ET may not be an option. The association of exercise, obesity and the prevalence of obesity in MetS, T2DM and comorbidities indicates the value in the contribution of this study with a very practical implication namely: Lifestyle-induced sedentariness is a major contributor to obesity and diabetes in western societies and efforts to increase general physical activity are mostly unsuccessful. These findings support the need for future investigations into types of exercise, inflammatory processes (and response) through the use of biomarkers and ratios and the hyperglycaemic state.
Acute Phase Inflammatory Response to Single-Bout HIIT and Endurance Training: A Comparative Study

Felix Kaspar1,2, Herbert F. Jelinek2,3*, Steven E Perkins2, Hayder A. Al-Aubaidy4, Bev de Jong2 and Eugene Butkowski2

1 Department of Biotechnology, Institute for Biochemistry and Biotechnology, Technical University Braunschweig, Braunschweig, Germany.
2 School of Community Health, Charles Sturt University, Albury, Australia.
3 Australian School of Advanced Medicine, Macquarie University, Sydney, Australia.
4 School of Medicine, University of Tasmania, Hobart, Australia.

* Corresponding author email: HJelinek@csu.edu.au

Abstract

Objective: The purpose of this study was to compare the acute and late effect of single-bout endurance training (ET) and high-intensity interval training (HIIT) on the plasma levels of five inflammatory cytokines and insulin-like growth factor 1 in a group of young untrained adults.

Design: Cohort study with repeated measures design

Methods: Seven healthy untrained volunteers (1 male, 6 females, 20.9 ± 0.9 yr, 69.5 ± 6.9 kg, 169 ± 6 cm) completed a single bout of ET (45 min at 62.5% of maximal heart rate) and HIIT (6 sets of 30 s supramaximal intensity separated by 4 min of recovery) on a cycle ergometer. ET and HIIT sessions were held in random order and at least 7 days apart. Blood was drawn before the interventions, 30 min and 2 days after the training sessions. Plasma samples were analyzed for IL-1β, IL-6, IL-10, MCP-1, IGF-1 and CRP via
commercially available sandwich ELISA kits. Wilcoxon signed-rank tests were applied to determine statistically significant results.

**Results:** ET led to both a significant acute and long-term inflammatory response with a significant decrease at 30 minutes post-exercise in the IL-6/IL-10-ratio (-20%; p = 0.047) and a decrease of MCP-1 (-17.9%; p = 0.03).

**Conclusion:** This study demonstrates that ET affects the inflammatory response more adversely at 30 minutes post-exercise compared to HIIT. However, this is compensated by a significant decrease in MCP-1 at two days associated with a reduced risk of atherosclerosis.

**Keywords:** INFLAMMATION, EXERCISE, EFFICIENCY, MCP-1, CYTOKINES, BIOMARKER

**Introduction**

Exercise and general physical activity are commonly associated with a healthy lifestyle and longevity as well as overall low levels of pro-inflammatory and high levels of anti-inflammatory cytokines (Petersen & Pedersen, 2005).

Cytokines of the interleukin group, including Interleukin 1β (IL-1β), Interleukin 6 (IL-6) and Interleukin 10 (IL-10), are key agents of the immune system and are involved in the systemic response to local inflammation (Petersen & Pedersen, 2005). Single exercise sessions of varying intensity and duration have been shown to induce systemic inflammatory responses similar to those associated with injury (Meckel et al., 2009; Ostrowski et al., 1998).
The acute-phase response (APR) is the body’s immediate response to inflammatory stimuli such as strenuous exercise and includes a complex mediator cascade aimed towards minimizing expansion of tissue damage and enabling recovery from pro-inflammatory processes. Exercise activates monocytes and macrophages with subsequent upregulation and expression of pro-inflammatory cytokines such as IL-1β, IL-6 and monocyte chemoattractant protein-1 (MCP-1) (Baumann & Gauldie, 1994; Shek & Shephard, 1998). MCP-1 levels, as well as those of the anti-inflammatory IL-10, have been shown to increase during and following exercise in order to contain inflammation and restore normal physiological function of the affected tissue (Hovanloo, Arefirad, & Ahmadizad, 2013; Ouyang, Rutz, Crellin, Valdez, & Hymowitz, 2011; Zwetsloot, John, Lawrence, Battista, & Shanely, 2014). IL-1β is also effective in the APR, pro-inflammatory cell activation and stimulation of APR gene expression (Baumann & Gauldie, 1994). Despite its principal role in the APR, findings of exercise-induced IL-1β increases have been inconsistent (Shek & Shephard, 1998). Since IL-1β increases not only its own production but that of IL-6 and C-reactive protein (CRP) as well, elevated levels of this cytokine may have adverse effects in the context of inflammation containment (Ganter, Arcone, Toniatti, Morrone, & Ciliberto, 1989a; Shek & Shephard, 1998).

Physiological levels of CRP levels are generally very low in healthy resting individuals, but can increase up to 10,000-fold during the APR (Pepys & Hirschfield, 2003). Previous studies on the relationship between single bouts of exercise and the CRP response have revealed conflicting data as a number of studies demonstrated post-exercise CRP level reductions, whereas others
failed to do so (Markovitch, Tyrrell, & Thompson, 2008; Michigan, Johnson, & Master, 2011). Further the physiological levels of anabolic hormones such as IGF-1, a pivotal mediator of muscle hypertrophy (Velloso, 2008), appear not to change following a single bout of endurance or resistance exercise (Meckel et al., 2009; Nindl et al., 2009).

High intensity interval training (HIIT) is a type of workout regimen that has recently become popular due to its time effectiveness compared to traditional more time-consuming forms of endurance training (ET). HIIT yields similar training results such as an increase in mitochondrial enzyme activity, muscle oxidative capacity and muscle glycogen content to conventional endurance training approaches by compensating the reduction in training volume with an increase in exercise intensity (Coyle, 2005; Gaesser & Angadi, 2011; Gibala et al., 2006; Zwetsloot et al., 2014).

Although the effects of single bout ET on inflammation have been extensively reported, studies on inflammatory responses to HIIT either investigated the systemic responses to repeated sessions (Hovanloo et al., 2013), examined HIIT in isolation (Zwetsloot et al., 2014) or in combination with other interfering stress factors including additional training volume and cumulative fatigue (Robson-Ansley, Blannin, & Gleeson, 2007). The overall consensus of these studies was that low-volume HIIT appears to lead to a similar inflammatory response to ET and supplies a sufficient training stimulus to generate significant physiological adaptations comparable to those achievable with endurance training in non-overreaching individuals.

To gain a better understanding of the acute and mid-term inflammatory responses to single exercise sessions and whether these may be related to the
type of exercise, we investigated the effect of single-bout HIIT and ET on five inflammatory cytokines and IGF-1 in a group of young untrained adults. We hypothesize that HIIT may cause a different inflammatory response compared to ET due to its larger impact on heart rate and highly exhaustive character.

Methods

All participants were informed of the testing and training procedures and the potential risks involved. Participation was fully voluntary and participants could withdraw from the study at any time. Written informed consent was obtained prior to study commencement. All participants were instructed to abstain from exercise for 48 h preceding and following the exercise intervention. Consumption of alcohol and caffeine was prohibited for the duration of the study. This study was approved by the Human Research Ethics Committee (Charles Sturt University, Albury, Australia), protocol number: 2014/161.

Nine healthy university students (Table 1) were recruited from student lectures two weeks prior to testing. Inclusion criteria were: age 18-30 years, body mass index: 18-30 kg·m⁻², blood pressure 90/60-140/90 mmHg, not taking any medications or supplements including anti-inflammatory drugs, not currently diagnosed with a chronic health condition, non-smoking and untrained (not training more than once per week at high or moderate intensity). Prior to the exercise bouts participants were screened to ensure they fit the inclusion criteria and were safe to undertake a high intensity
exercise program in accordance with the Exercise and Sports Science Australia adult Pre-Exercise Screening Tool (2011) stages 1 and 2.

TABLE 1: Subject

<table>
<thead>
<tr>
<th></th>
<th>20.9 ± 0.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td></td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>1/6</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69.5 ± 6.9</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>169 ± 6.0</td>
</tr>
<tr>
<td>Body Mass Index (kg·m⁻²)</td>
<td>24.4 ± 2.3</td>
</tr>
<tr>
<td>Blood glucose level (mmol·L⁻¹)</td>
<td>5.1 ± 0.8</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>116.7 ± 7.2</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>70.4 ± 4.7</td>
</tr>
<tr>
<td>HDL (mmol·L⁻¹)</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>LDL (mmol·L⁻¹)</td>
<td>2.6 ± 0.8</td>
</tr>
<tr>
<td>Total Cholesterol (mmol·L⁻¹)</td>
<td>4.5 ± 1.0</td>
</tr>
<tr>
<td>Triglycerides (mmol·L⁻¹)</td>
<td>1.1 ± 0.3</td>
</tr>
</tbody>
</table>

Data is presented as mean ± SD,
Abbreviations:
SBP: systolic blood pressure
DBP: diastolic blood pressure
HDL: high density lipoprotein
LDL: low density lipoprotein

After completing the preliminary screening, participants were familiarized with the equipment used for the exercise protocols. Participants were randomly assigned either to perform the ET or the HIIT protocol first at least three days following the familiarization. The ET and HIIT sessions were performed at least seven days apart and in the morning (between 8 am and 12 noon). This 7-day wash-out period between the sessions was applied to ensure cytokine levels had returned to baseline before beginning the second intervention based on results by Ostrowski et al. (Ostrowski et al., 1998), who describes 5 days to be sufficient for cytokine levels to normalize.
The ET and HIIT sessions consisted of single supervised morning exercise sessions performed on an air-braked cycle ergometer (Wattbike Ltd, Nottingham, UK).

The HIIT session involved a 2 min warm up at <50 watts followed by 6 sets of 30s of all-out supramaximal intensity cycling at the participant’s respective self-selected gearing. Between sets participants completed a 4 min recovery period in which they either rested or cycled below 30 watts.

The ET session consisted of 45 min of ergometer cycling at a moderate intensity, which was calculated at 62.5% of maximal heart rate. Maximum heart rate was calculated according to the formula suggested by Tanaka et al. (208 – 0.7∙age) (Tanaka, Monahan, & Seals, 2001). Heart rate was measured by a heart rate monitor (RS800CX; Polar Electro Ltd.) with chest strap. Participants were able to monitor their current heart rate and were asked to maintain constant exercise intensity at their calculated heart rate.

Baseline blood samples were taken after 5 min of rest in a seated position on the day of the first intervention. Post-exercise blood samples were taken 30 min and 2 days after completion of the ET and HIIT protocol. At each time point, 20 mL of blood was drawn from the antecubital vein in two 10-mL EDTA-tubes, centrifuged for 15 min at 800 g and 4°C. The plasma was transferred into 2 mL Eppendorf tubes (Eppendorf AG, GER) and stored at -80°C until analysis (within 4 months).

Each sample was analyzed in duplicate using commercially available sandwich ELISA kits (elisakit.com, Scoresby, VIC) in compliance with the supplier’s instructions. According to the information provided by the manufacturer, the lower limit of quantification of the assays for IL-1β was
less than 1 pg/mL\(^{-1}\), less than 5 pg/mL\(^{-1}\) for IL-6, IL-10, IGF-1 and less than 10 pg/mL\(^{-1}\) for MCP-1. The intra- and interassay coefficient of variance was <10% for all assays. The Lot numbers were: IL-1β: #P-150702; IL-6: #L-150422; IL-10: #150409; IGF-1: #150612; MCP-1: #P-150417. The optical density at 450 nm was measured with a Multiskan FC Microplate Photometer (Thermo Fisher Scientific Inc., Waltham, MS).

Serum CRP levels and cholesterol profile were provided by Dorevitch Pathology Laboratory, Albury, NSW. Blood Glucose levels were measured by BGL meters (Hoffmann-La Roche, Basel, GER) from finger blood samples.

Data were analyzed with Microsoft Excel (Office 2010, Microsoft), SPSS (Version 22, IBM Inc.) and S-Plus 8 (TIBCO, Seattle, Washington). Wilcoxon signed-rank tests were applied to determine if there were significant changes in the biomarker levels for post-ET and post-HIIT and between ET and HIIT. Correlation analyses were performed with Origin (OriginLab, Origin ProLab 9) and SPSS. A p-value <0.05 was considered as significant. To determine the association between CRP, IL-6 and IL-1beta the synergistic model recommended by Ganter et al. was used (Ganter et al., 1989a). Descriptive statistics for continuous variables were calculated in EXCEL and are expressed as mean ± SD. Immunoassay data are presented as mean ± standard error.

**Results**

Data from two of the initial nine participants were excluded from the analyses. One participant could not complete the HIIT protocol. Hemolysis impeded cytokine measurements of the second participant’s samples.
There were no significant changes in the plasma levels of CRP, IL-1β, IL-6, IL-10 and IGF-1 from baseline to either 30 min or 2 days after the intervention (Table 2). However, there was a significant decrease in the IL-6/IL-10-ratio from baseline to thirty minutes post-ET (-20%; p = 0.047). MCP-1 concentrations also decreased significantly from baseline to 2 days post-ET (-17.9%; p = 0.03). These changes reflected at the 30 min mark where a trend towards a significant difference between the post-ET and post-HIIT change in CRP (ET: 3.29 ± 1.01 > HIIT: 2.07 ± 0.69; p = 0.1) and IL-6 (ET: 27.25 ± 19.38 < HIIT: 37.76 ± 19.49; p = 0.09) was observed.

**TABLE 2: Blood marker data prior to, 30 min and 2 days after ET and HIIT**

<table>
<thead>
<tr>
<th></th>
<th>CRP*</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-10</th>
<th>MCP-1</th>
<th>IGF-1</th>
<th>IL6/IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRE</td>
<td>3.00 ± 1.02</td>
<td>42.32 ± 5.30</td>
<td>30.40 ± 14.72</td>
<td>113.25 ± 24.42</td>
<td>224.83 ± 17.12</td>
<td>345.28 ± 70.34</td>
<td>0.34 ± 0.13</td>
</tr>
<tr>
<td>ET</td>
<td>3.29 ± 1.01*</td>
<td>38.61 ± 5.50</td>
<td>27.25 ± 19.38*</td>
<td>109.16 ± 36.02</td>
<td>187.20 ± 27.90</td>
<td>302.37 ± 47.48</td>
<td>0.27 ± 0.14**</td>
</tr>
<tr>
<td>30 min</td>
<td>2.92 ± 1.07</td>
<td>45.36 ± 5.10</td>
<td>33.94 ± 21.61</td>
<td>97.28 ± 28.80</td>
<td>184.68 ± 17.36*</td>
<td>308.80 ± 58.24</td>
<td>0.50 ± 0.29</td>
</tr>
<tr>
<td>2 days</td>
<td>2.07 ± 0.69*</td>
<td>37.47 ± 4.34</td>
<td>37.76 ± 19.49*</td>
<td>97.47 ± 24.94</td>
<td>215.52 ± 24.75</td>
<td>298.98 ± 34.43</td>
<td>0.41 ± 0.16*</td>
</tr>
<tr>
<td>HIIT</td>
<td>1.90 ± 1.81</td>
<td>39.49 ± 6.19</td>
<td>38.94 ± 22.64</td>
<td>82.95 ± 37.58</td>
<td>165.38 ± 21.85</td>
<td>256.16 ± 76.80</td>
<td>0.55 ± 0.25</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SE
*CRP concentrations are given in mg·L⁻¹; all other concentrations are given in pg·mL⁻¹
*Trend towards significance at 30 min post-exercise between ET and HIIT for CRP (P = 0.1), IL-6 (P = 0.09), and the IL-6/IL-10 ratio (P = 0.08)
*Significant changes for MCP-1 PRE to 2 days post-ET (P = 0.03) and for IL-6/IL-10 ratio for PRE to 30 min post-ET (P = 0.047)

A significant correlation between CRP and a calculated score based on IL-1β and IL-6 levels (Pearson R = 0.63; p = 0.01) was also observed. This score was calculated from the synergistic model of Ganter et al. using IL-6^IL-1β
and normalized values of all patients from all points in time (Ganter et al., 1989a).

**Discussion**

This is the first study to directly compare the inflammatory response to single-bout ET and HIIT in the same cohort as well as investigate acute-phase response and medium-term response of cytokines to single-bout HIIT and ET. Our cohort was comparable in numbers to previous publications but used a repeated-measures design to obtain a better statistical power.

Endurance training reduces MCP-1 levels and has been associated with a reduction in the development of atherosclerosis, metabolic syndrome and diabetes by acting on visceral fat reserves. MCP-1 and has also been shown to be reduced following moderate levels of exercise in heart failure patients (Adamopoulos et al., 2001; Boring, Gosling, Cleary, & Charo, 1998). Therefore we sought to determine whether MCP-1 is reduced in young adults undertaking HIIT and whether this differs to results reported for ET. MCP-1 concentrations decreased after both training sessions and significantly decreased two days post-ET. Zwetsloot et al. (Zwetsloot et al., 2014) showed that MCP-1 levels were significantly elevated immediately after exercise and Sugama et al. (Sugama, Suzuki, Yoshitani, Shiraishi, & Kometani, 2013) demonstrated a high flush-out rate of this protein into urine. Therefore our measured decreased levels may be lower following ET and HIIT due to the washout effect. However the decreased MCP-1 levels two days post-ET and post-HIIT may also be due to a long-term decline in oxidative stress (Troseid et al., 2004). MCP-1 levels continued to decrease further following HIIT, which may be due to a more effective reduction in oxidative stress in addition
to reported increased levels of lactic acid following HIIT (Peake et al., 2014; Peter, Rehli, Singer, Renner-Sattler, & Kreutz, 2015). In the context of beneficial effects of single-bout ET and HIIT, one major health benefit of maintaining generally low levels of MCP-1 is the reduction of risk of cardiovascular disease and diabetes (Deshmane et al., 2009).

Our initial hypothesis that inflammatory responses would be different following single-bout ET versus single-bout HIIT was confirmed by a trend towards a greater decrease in the pro-inflammatory CRP and an increase in IL-6 levels following HIIT at thirty minutes following exercise. Neither training protocol appeared to cause significant acute changes of any single inflammatory cytokine possibly due to the relatively short training period (HIIT: 25 min, ET: 45 min). Previous investigations with shorter single-bout exercise duration (30 min) and lower intensity (50% maximal oxygen uptake) reported a comparable inflammatory response (Markovitch et al., 2008).

Previous work by Kasapis et al. (Kasapis & Thompson, 2005) reported consistent increases in CRP levels after very strenuous exercise including marathons. The CRP decrease after HIIT in the present study is in agreement with the findings of Hovanloo et al. (Hovanloo et al., 2013) who demonstrated a small CRP decrease 48 h after completing six HIIT sessions at a less strenuous level compared to Kasapis et al..

A slight post-HIIT increase in IL-6 levels, which is in agreement with the much of the literature, was observed (Bernecker et al., 2013; Ostrowski et al., 1998; Zwetsloot et al., 2014). These non-significant increases may be due to the short duration or lower exercise intensity, which did not affect muscle physiology enough to lead to a significant increase in IL-6 levels (Tomiya, 188
Aizawa, Nagatomi, Sensui, & Kokubun, 2004). Single-bout ET and HIIT therefore have minor effects on muscle tissue integrity that non-the-less may lead to a greater reduction in CRP levels following HIIT by IL-6 acting as pro-inflammatory cytokine (Petersen & Pedersen, 2005).

IL-6 is also the main inducer of CRP transcription but requires the presence of sufficient IL-1β in this regard. Although the presence of IL-6 by itself significantly raises CRP transcription, maximal expression only occurs when both cytokines are active (Ganter et al., 1989a). Since IL-1β levels decreased post-HIIT, which is in agreement with previous findings by Zwetsloot et al. (Zwetsloot et al., 2014), we employed a mathematical model to simulate a synergetic action of IL-6 and IL-1β on CRP expression as previously suggested by Ganter et al. (Ganter et al., 1989a). The regulatory role of IL-1β in this context helps to explain why post-HIIT CRP levels decreased in spite of elevated IL-6 levels in our study. However, future studies will be required to validate this model.

No significant change in IL-10 levels was observed, although a slight decrease was noted, which is, in agreement with the findings of Hovanloo et al. (Hovanloo et al., 2013) and Zwetsloot et al. (Zwetsloot et al., 2014) regarding HIIT. Our findings for the ET intervention are inconsistent with the literature as most studies reported marked post-exercise increases of IL-10 in contrast to our study showing no effect (B. K. Pedersen, 2000; Sugama et al., 2013). However, these followed much more exhausting bouts of exercise. The recent study by Markovitch et al. (Markovitch et al., 2008), which resembles our ET protocol more closely, did not detect a significant IL-10 change. The observed decrease of plasma IL-10 levels could be attributed to a similar
mechanism found for the MCP-1 decrease as Sugama et al. (Sugama et al., 2013) also reported a rapid increase of IL-10 in the urine 1.5 h post-exercise. Given that the first post-exercise blood sample was taken 30 min after the intervention and no additional IL-10 release, it is possible that the original levels of IL-10 had already declined due to increased post-exercise water turn-over.

Although mean IGF-1 levels decreased after both ET and HIIT there was no significant change to be noted. The findings are consistent with the results by Nindl et al. (Nindl et al., 2009) who demonstrated that IGF-1 levels do not increase or decrease significantly after moderate or long duration aerobic or anaerobic exercise. The present study adds to this body of evidence and shows that circulating total IGF-1 levels do not change significantly after single bouts of ET or HIIT.

Regular aerobic exercise such as ET improves general health. HIIT, however, can produce similar results to ET including muscle oxidative capacity and mitochondrial enzyme activity, but in addition to providing various cardio metabolic benefits HIIT is also very time-efficient (Gaesser & Angadi, 2011). The lack of large-scale post-HIIT or post-ET inflammatory responses indicates that a regimen of two to three HIIT or ET sessions per week may noticeably aid to improve general health and fitness such as body fat percentage, maximal oxygen uptake and cardiovascular endurance while exerting relatively little stress on the immune system in untrained and healthy young adults (Gibala et al., 2006).
Conclusions

The novel findings of the present study are that 1) there was no significant difference between HIIT and ET in regards to individual cytokine responses and 2) IL-6 may be acting as an anti-inflammatory cytokine that leads to a greater decrease in CRP following HIIT and similar to ET a decreased MCP-1. These results support the hypothesis that HIIT may present a valid alternative to ET for individuals that are short on time or personally prefer a HIIT-type regimen to achieve equivalent overall health benefits to ET.

Practical Implications

- Lifestyle-induced sedentariness is a major contributor to obesity and diabetes in western societies and efforts to increase general physical activity are mostly unsuccessful. Therefore, various exercise protocols should be assessed.
- The present study shows that there is no significant difference between the inflammatory response to conventional endurance training and high intensity interval training and that neither protocol caused severe acute cytokine increases.
- Time-efficient high intensity interval training, therefore, should be considered a valid alternative to conventional training approaches to improve general health and fitness.

Acknowledgements

The authors thank all volunteers for their participation and Hoffmann-La Roche for providing the Blood Glucose strips and acknowledge the highly
valuable contribution of Simon McDonald of conducting the non-parametrical statistical analyses. Helpful suggestions on a previous draft were made by Dr. Ian Spence.

The authors have nothing to disclose and no conflict of interests.

References


Discussion

At the commencement of this study the question asked was if there have been screening programs available for the future risk of CVD in diabetic patients, then why are the numbers of CVD patients increasing knowing also that T2DM is steadily increasing at the global level? If the number of T2DM is increasing then clearly predictive measures for risk stratification is not supplying the entire information. In 1948 the Framingham Heart study program commenced to identify risk factors for heart disease. The surveillance of subsequent cardiovascular events prior to evidence of diabetes revealed that the risk of the development of atherosclerosis increased by up to three fold (Kannel & McGee, 1979). Our initial hypothesis offered an alternative model of macrovascular assessment which involved the proposed investigation of oxidative stress utilising the biomarkers which had previously shown significant differences in patients with DM and/or pre-diabetes: GSH levels with established CVD compared with those that had either established DM or prediabetes was significant (p <0.0001). D-dimer levels were highest in DM patients (p <0.003), but increased in pre-DM with CVD (p <0.007). MDA and Hcy were also significant in the prediabetic state (p =0.05 and p <0.01 respectively). As our hypothesis involved the recognition that hyperglycaemia induced GSH depletion is linked to macrovascular complications (Nwose, Richards, et al., 2009), D-dimer an indicator of thrombotic activity was also selected. Previous correlations of GSH with D-dimer in T2DM indicated that there is a correlation with oxidative stress and coagulopathy (E. U. Nwose, H. F. Jelinek, R. S. Richards,
The object of this study was to provide an alternative improved modelling in screening subclinical diabetes to obtain better identification of those patients that would not qualify for interventional treatment.

Our hypothesis placed a strong emphasis on hyperglycaemia and oxidative damage as an underlying mechanism in macrovascular pathogenicity. It was noted that an array of anthropometric, traditional and emerging biomarkers may reflect different biochemical processes and not necessarily the same pathophysiological pathways. A further recommendation was to run larger sample sizes and perform a longitudinal analysis in order to refit lipid modelling and tailor to a more exclusive model for CVD risk in subclinical diabetes. On this basis our recommendation was to use glucose as a continuous variable and a categorised oxidative stress biomarker such as GSH and a coagulant biomarker D-dimer.

In paper 2, following through on the hypothesis, this study examined the balance between GSH and its oxidised form glutathione disulfide (GSSG) and any observable disturbance relevant to impaired fasting glucose (IFG). Oxidative stress is implicated in DM, obesity, atherosclerosis, coronary heart disease, heart failure and renal disease. Metabolic syndrome (MetS) may be a common link to many of these disorders due to the presence of hyperglycaemia induced oxidative stress (H. F. Jelinek et al., 2014). As this study was observing oxidative stress, 8-OHdG was employed to enable a more comprehensive picture of the redox state. Standard markers such as glucose, HbA1c and cholesterol were also used. IFG as a preclinical state of
T2DM is a useful target for early intervention. We based this study cohort on increased glucose levels, but not diabetic status, and selected patients without any evidence of CVD, hypertension, renal dysfunction and were not medicated. Apart from glucose (p < 0.001) traditional biomarkers did not reveal any significant difference between the control and IFG. GSH, GSSG and 8-OHdG were not significantly decreased or increased respectively. A very important finding was the significant decrease in GSH:GSSG ratio between the control group and the IFG group (p < 0.05). It was postulated that the decreased GSH:GSSG ratio may be indicating an early thiol related OS response and impaired redox status with erythrocyte GSH levels slightly up but similar to control levels. This accords with previous studies (Hayden & Tyagi, 2002; Vijayalingam, Parthiban, Shanmugasundaram, & Mohan, 1996). The significance of the reduced GSH/GSSG redox balance does indicate oxidative stress and may therefore be a robust marker of OS associated with IFG, with a normal cholesterol profile. Elevated 8-OHdG have been shown to be elevated in IFG but this requires an increase in triglycerides. The relationship between 8-OHdG and cholesterol is controversial with differing positive and negative correlations (Al-Aubaidy & Jelinek, 2014; Kikuchi et al., 2013; Nwagha, Ikekeazu, Ejezie, Neboh, & Maduka, 2010; Sakano et al., 2009).

The results of this study provided some valuable insight into oxidative stress in IFG. Whilst the decrease in GSH was not significant and likewise GSSG was not increased our findings would indicate the significant GSH:GSSG ratio may reflect more sensitivity in oxidative stress changes and its
robustness as an early indicator of OS in IFG. Paper 3 built upon and expanded upon correlations between oxidative stress and inflammatory markers and applied it to T2DM and the Framingham CVD risk score. Conflicting results on the use of oxidative stress biomarkers may indicate their lack of association with T2DM and CVD disease progression. In addition to the traditional and oxidative stress biomarkers interleukin 6 (IL-6), a pro-inflammatory marker was included in this study. T2DM carries a risk of two to six fold risk of CVD and atherosclerosis may precede T2DM indicating there are multiple independent pathways to CVD and atherosclerosis (P. Libby, Ridker, & Hansson, 2009). The link between OS and the inflammatory process affects a multitude of cellular responses in organ systems and progression of insulin resistance is known to be associated with inflammation and OS (Vikram, Tripathi, Kumar, & Singh, 2014) therefore the IL-6 was included as an inflammatory marker in our investigation.

The oxidative and inflammatory emerging biomarkers quantified in this study were GSH, GSSG, GSH/GSSG, IL-6 and traditional biomarkers were lipids, HbA1c, CRP and glucose. Significant differences were identified for IL-6 (p <0.05) between the control and T2DM and prediabetic and T2DM group and GSH/GSSG (p <0.05) between the control and pre-diabetic group, with results decreasing between prediabetic and T2DM group. IL-6 was significant between the low and moderate CVD risk group.

The results obtained in this study affirmed the importance of incorporating emerging OS and inflammatory biomarkers when considering indicators of
T2DM progression. GSH differed significantly between CVD risk and the diabetic groups. GSH was significantly higher in the moderate CVD risk compared to low risk and significantly decreased in the high risk. This increase followed by a decrease was not observed in the diabetic subgroups suggesting that there are differing antioxidant activities with respective pathologies. The increase in GSSG and return to normal in both diabetic groups indicated a redox cyclic change. The GSH:GSSG ratio also confirmed previous findings (H. F. Jelinek et al., 2014) indicating it may be more sensitive as marker than just GSH, however this could also reflect the higher levels of GSH normally present when compared to GSSG. Increased erythrocyte OS observed in T2DM and CVD risk results in the up regulation of procoagulant factors and proinflammatory mediators which would then contribute to endothelial dysfunction evidenced in atherosclerosis.

IL-6 levels were significantly increased in the T2DM group when compared to the control and the preDM group, whereas the CVD risk group had a significant difference between the low and moderate risk CVD group, however caution is still required when interpreting IL-6 levels as a previous study has shown high levels are associated with obesity and insulin resistance and do contribute to the development of CVD (Dandona, Aljada, & Bandyopadhyay, 2004). If IL-6 is also associated with macrovascular complications in T2DM interpretation may be difficult as T2DM is considered a risk factor for CVD. Further investigations into IL-6 to assess whether levels independently assist in prediction of macrovascular events need to be conducted. Supportive evidence that increased levels of IL-6 have
been correlated with the development of atherosclerosis in T2DM (W. Wu et al., 2012) warrant the continued investigation of IL-6 and the hyperglycaemic state.

In paper 4 the study into biomarkers and their association with increasing blood glucose levels (BGL) were divided into 1st and 4th, 5th quintiles (4.5mmol/L vs >6.1mmol/L respectively). In this study the recognition that OS and inflammation emerging from and participating with the pro-inflammatory hyperglycaemic state is not fully understood. The previous paper demonstrated OS (GSH, GSH:GSSG) and inflammation (IL-6) was disturbed in hyperglycaemia. This study provided further confirmation. The study population in this group was from a diabetic screening clinic so patients were not discriminated and excluded based upon medical and medication history. Anthropometric data was obtained and blood collected for analysis. The OS biomarkers analysed in this study were: GSH, GSSG, 8-OHdG. As we had previously demonstrated that IL-6 provided significant results the inflammatory biomarker regime was expanded to include: IL-1β, IL-6, IL-10, and MCP-1. Insulin like growth factor 1 (IGF-1) was also included as an emerging biomarker as it is postulated to be lower in the inflammatory state and glucose intolerance (Rajpathak et al., 2008). Our results for IGF-1 were not conclusive and will require further investigation as lower levels are also associated with aging and frailty (Maggio et al., 2013). The comparison of oxidative stress and inflammatory biomarkers compared the 1st and 4th, 5th quintiles. GSH (p <0.001) was decreased significantly between quintile groups indicating a state of oxidative stress.GSH/GSSG, 8-OHdG and the
inflammatory marker IL-1β were increased (p <0.05). This supports the concept of monitoring the state of oxidative stress during prolonged hyperglycaemia, as a consequence is the increased risk of micro and macrovascular complications (Sekhar et al., 2011), considering also the inflammatory component as seen with the significant increase in IL-1β. It is possible that the increased ROS is contributing to the structural and functional damage of endothelium, a factor in the development of atherogenesis, and evidenced by increased IL-1β (Lee, Kim, Lee, & Hirani, 2010). Previous studies have disputed a link between OS and hyperglycaemia and oxidative stress (Choi et al., 2008). This study however has shown for the first time a link with 8-OHdG and a number of possible inflammatory biomarkers interacting with inflammatory markers including IL-1β/IL-10. Elevated levels of IL-1β have been associated with hyperglycaemia, insulin resistance and, obesity – all factors evident in the development T2DM (Koenen et al., 2011). A recent study on IL-10 has provided further evidence that levels decrease in insulin resistance (Leon-Cabrera et al., 2015) supporting our findings of an increased IL-1β/IL-10 ratio between the quintile groups. While IL-10 results were not significant the ratio IL-1β/IL-10 was, indicating IL-10 may not be responsible for smaller increases in BGL but lower levels of BGL may still influence IL-1β activity. CRP/IL-6 was investigated as research has shown an increase in CRP and IL-6 activity in diabetes (Schöttker et al., 2013). Our findings on CRP/IL-6 in this study demonstrated a decrease in the higher quintile group, indicating that CRP increase may well be a function of IL-6 in the inflammatory process. Studies by Arnalich et al. on whether oxidative stress could promote a systematic acute-phase response in elderly
patients with T2DM have found that multiple regression analysis of markers such as lipid peroxidase and IL-6 correlated independently with CRP; suggesting oxidative stress might be implicated in promoting a state of low-grade systemic inflammation in their patient cohort (Arnalich et al., 2000).

This study therefore highlighted the value of incorporating these emerging biomarkers as part of a general screening of hyperglycaemic clients irrespective of their treatment regimen as findings did replicate well controlled experiments.

An outcome of paper 4 was the observation that a number of biomarkers tested on a screening population replicated results of well controlled experimental analysis. As we had established emerging biomarkers may be useful as a screening tool for hyperglycaemic patients in an outpatient clinic this study cohort reviewed medication use in MetS. Patients were classified with MetS if three or more factors were present according to the National Education Program Adult Treatment Panel III (ATP III). Patients with MetS have a fivefold chance of developing T2DM and a fivefold chance of developing atherosclerotic CVD. Antidiabetic (Dmeds), antihypertensive (HT) and statin were compared in no MetS vs MetS groups. Medication increased with the degree of hyperglycaemia, for example Dmeds tripled in medication type use in the MetS groups (p <0.001). However Dmeds were not found to be prescribed in patients with a BGL >6.1mmol/L but less than 7mmol/L, whereas HT and Statins were extensively used when only 1 – 2 MetS factors were present. The Framingham Risk Study (FRS) states that vascular disorders are central to MetS as 80% of men and 65% women with
hypertension are obese (Garrison et al., 1987). Prophylactic statin use is warranted and may provide efficacy even in patients with normal cholesterol as suggested by outcomes from the Heart Protection Study Collaboration (HPS) and the Collaborative Atorvastatin Diabetic Study (CARDS) (Colhoun et al., 2004; Collins, Armitage, Parish, Sleigh, & Peto, 2003). The biggest increase when comparing no MetS to MetS (<3 vs ≥3) was the use of Dmeds suggesting the incidence of T2DM may be strongly related to the increase in obesity, blood pressure and cholesterol levels.

This study indicated that in a focused community MetS is relatively well controlled and the majority of risk factors for CVD are below the threshold level but we did observe waist circumference remains higher and was significant in women (p <0.01). The recognition of the importance of lifestyle changes as a primary way of managing MetS and addressing such factors as smoking, alcohol use and physical exercise is important but in the presence of MetS prescribed medication is important as lifestyle changes may not be sufficient to ameliorate MetS. We did demonstrate that Statin use may also be below that recommended as the increased systolic blood pressure and HT category (5 MetS) was quite high suggesting there is a higher risk of CVD in this population.

To expand our understanding on the impact of emerging biomarkers into hyperglycaemia we next investigated the role of Hcy, a marker for endothelial dysfunction. The goal was to establish if Hcy provided any contributory role in the development of MetS.
As endothelial dysfunction arises due to OS and inflammatory disturbances, in paper 6 we looked at the role of Hcy and the OS biomarkers GSH and 8-OHdG and the inflammatory biomarker IL-6 in relation to MetS. It was decided to include patients on medication to determine whether Hcy results could be used as a risk indicator for T2DM and CVD in community health screening programs as previous results obtained were indeterminate. Elevated Hcy promotes atherosclerosis through increased OS, impaired endothelial function and the induction of thrombosis. OS and inflammation are mechanisms associated with one or more of the 5 MetS factors, T2DM and CVD (Ridker et al., 2001; Russell Ross, 1999; Skibinska et al., 2004). The importance in understanding the impact of Hcy on MetS was due to OS and inflammatory processes being linked through Hcy. Due to the association of Hcy with increased insulin resistance, increased blood pressure (BP) and increased LDL, constituting 3 out of the 5 MetS factors an important question was to which cluster of MetS factors is Hcy associated with.

We observed significant results in no MetS and MetS with anthropometric and traditional markers including waist circumference (WC), Systolic BP, TC, TG, HDL, LDL, BGL and HbA1c. Hcy and 8-OHdG were significantly elevated in the MetS group compared to the no MetS (p <0.001). MDA, a general OS marker was significant between 2 MetS and 4 MetS (p =0.01) and CRP was significant between 0 MetS vs 1 MetS and 3 MetS (p <0.5). The ATP III definition of MetS was applied and included in the study was patients receiving glucose lowering, HT and lipid lowering medications. Our reasoning for this was to concentrate on a population sample for assessment of Hcy as a clinically usable indicator. Defining MetS does not include factors
such as age, gender, LDL and emerging factors such as OS, inflammatory and coagulation biomarkers (H. F. Jelinek et al., 2014; H. F. Jelinek et al., 2015; L. Maschirow et al., 2015). A hallmark of endothelial dysfunction is CVD and T2DM so our suggestion would be the inclusion of the emerging biomarkers in risk assessment, in conjunction with assessment according to the MetS criteria. Hcy along with 8-OHdG may be useful markers, recognising our results did confirm that Hcy is not correlated with 8-OHdG (Kuwahara et al., 2013). However, GSH increased up to four MetS factors present, then decreased dramatically followed by an increase when five MetS factors were present. Was this an artefact or could it possibly result from the downregulation of Hcy? We have demonstrated this phenomena in previous studies so our suggestion was that GSH displays a cyclic change with disease progression, firstly increasing as OS increases, but then decreasing as a result of the OS prior to a de novo synthesis leading to a secondary increase in GSH (Butkowski et al., 2016; H. F. Jelinek et al., 2014). This secondary increase in GSH is further enhanced due to a decrease in Hcy which was observed in this study and may be a consequence of increased medication (D. E. Handy, Y. Zhang, & J. Loscalzo, 2005).

We concluded that the significant results associated Hcy and the clustering of MetS factors may be the result of complex pathophysiological metabolic interactions with GSH and 8-OHdG, suggesting that pathological vascular processes are more pronounced with the increasing MetS factors. (Figure 1 in paper 6 depicts the trending of Hcy, GSH and 8-OHdG).
As this thesis was developing a constant scrutiny of the efficacy of topical emergent biomarkers to support predictability and prevention of T2DM and CVD a publication emerged stating that lower levels of creatinine may be a predictor of T2DM. To test this theory we utilised archived and current pathology data to examine if lower levels of creatinine as a risk factor for diabetes could be supported. We further extrapolated the claim to include the prediabetic state to observe if any significance could also be attached. If so, a continuum would be observed. As this was a pathology laboratory database an eGFR was automatically generated. Of 1016 glucose tolerance tests (OGTT) conducted four categories premised upon the decisive interpretation (Harita et al., 2009). Once the data was filtered to include OGTT and creatinine 102 patients were classified as control group, Prediabetes and Diabetes. Creatinine results (and eGFR) indicated no significant differences between any of the groups. As a comorbidity of T2DM is renal nephropathy, decreasing renal function would generally see an increase in circulating levels of creatinine, as would a decrease in eGFR. Due to the lack of any statistical significance of creatinine between the groups it was concluded that diabetes pathogenesis is unassociated with low serum creatinine levels. However this was not a large sample so it was suggested that these findings be tested with a larger cohort.

Realising the value of access to a large data base of pathology, a number of publications were presented to support the use of archived pathology data which is readily assessable and available for scrutiny. These co-authored publications are listed in the appendices as appendix - A, B, D, E and F.
The purpose of the final 3 papers presented in this thesis was to examine the emerging biomarkers with comorbidities associated with T2DM. In paper 8 OS, inflammatory and coagulation markers were assayed in conjunction with the ankle brachial pressure index (ABPI). A number of factors known to influence the formation of atherosclerosis are coagulation, fibrinolysis, hyperglycaemia, hyperlipidaemia and hyperinsulinaemia. Having established that OS, inflammation and coagulation biomarkers are emerging as useful indicators in the development of T2DM and CVD a logical extension was to view their association with the comorbidities of T2DM. A significant proportion of diabetics suffer from hypertension and likewise hypertensive patients may demonstrate insulin resistance. As peripheral vascular disease (PVD) is a comorbidity of T2DM, ABPI was conducted in conjunction with emerging biomarkers. The reliance upon ABPI alone for PVD diagnosis is problematic as it is limited due to calcification of larger arteries (Scanlon, Park, Mapleton, Begg, & Burns, 2012). Foot ischaemia due to atherosclerosis and atherosclerosis as a consequence of vascular calcification are suggestive of differing pathologies so a challenge was to select biomarkers which may discriminate between the two. Patients studied in this cohort were selected from the diabetic screening clinic and classified on the basis of an ABPI tertile groups of less than 1.07 and above 1.23 respectively. Emerging biomarkers analysed were D-dimer (D-dimer relevant to the context of this study, like Hcy is categorised as emerging), IL-6, GSH, MDA, 8-OHdG and CRP. As these analytes were not normally distributed, a finding in accordance with previous observations (G. D. O. Lowe, Yarnell, Rumley, Bainton, & Sweetnam, 2001; Salomaa et al., 1995) the biomarkers underwent log
transformation prior to modelling. The traditional biomarkers were not significant. The atherogenic index of plasma (AIP) whilst normal in the low ABPI indicated that there was a greater risk of atherosclerosis and CVD. Emerging biomarkers associated with an increasing risk of CVD, including PVD and diabetes showed only D-dimer (p <0.001) and GSH (p = 0.024) as significant between low and high ABPI. The interaction between fibrinolysis and the level of GSH had been previously established so we also investigated possible models for classification of ABPI which included only traditional biomarkers or the inclusion of emerging biomarkers. The results indicated the best model to predict ABPI class was the inclusion of the emerging biomarkers: CRP, D-dimer, and IL-6 combined with BGL, HbA1c and SBP (p <0.001) as compared to the best traditional biomarker model: HbA1c, AIP, LDL and SBP (p <0.05). These results confirmed that their inclusion enable better discrimination between low and high ABPI as a decreased inflammatory component with increased OS would be more likely to lead to arterial calcification and a higher ABPI, despite normal BGL, HbA1c and lipid profile, thus confirming that emerging biomarkers provide discriminating information and suggest differing pathophysiological pathways.

The study undertaken in paper 9 investigated OS and inflammatory markers associated with cognitive decline, a comorbidity of T2DM. As the association with cognitive decline and T2DM is inconclusive we investigated patients with the Stroop battery of tests consisting of the reading interference test (RIT) and the naming interference test (NIT) and then tested for any
association with an inflammatory response. Mild cognitive impairment (MCI), a common age related phenomena, which may be a pre-clinical stage of dementia has been linked to obesity, vascular disease, dyslipidaemia, inflammation and oxidative stress (N. Lorius et al., 2015; M. W. Marlatt, Lucassen, Perry, Smith, & Zhu, 2008; J. C. D. Nguyen, A. S. Killcross, & T. A. Jenkins, 2014). Oxidative stress and inflammation associated with vascular disease is also a consideration recognising that oxidative stress and inflammatory cytokine production are intricately linked (A. A. Elmarakby & Sullivan, 2012). Participants for this study, all >40 years of age, were recruited from a rural health screening clinic for cognitive assessment and divided into two groups based on the normal range for Stroop. Inflammatory cytokines analysed in this study were CRP, IL-1β, IL-6, MCP-1, and anti-inflammatory cytokine IL-10 and the growth factor IGF-1. Results were divided into low and high Stroop score for RIT and NIT. Establishing a cut-off Stroop score by setting a t-score of 60, representative of 1SD from the normalised mean classified a normal cognitive score. Comorbidities were defined as BGL >7.0mmol/L, hypertension as either self-reported with or without medication, or SBP >120mmHg. CVD was identified with a 12 lead ECG or self-reported. Results obtained showed CRP was significant for RIT (p = 0.022); IL-1β (p = 0.039), MCP-1 (p <0.01) were significant for NIT. IL-10 (p <0.01, IL-6/IL-10 (p <0.03) were significant in RIT and NIT. Current research indicated that any cytokine association with Stroop would depend upon whether the RIT or NIT was applied. This study used RIT and the NIT to categorise high and low Stroop scores and investigated if pro or anti-inflammatory cytokines were associated with Stroop score. NIT and RIT
scores were not correlated with the same inflammatory biomarkers tested. IL-10 and the ratio IL-6/IL-10 were consistent between the two Stroop scores. As IL-1β was increased in NIT and not RIT, this indicates the need to be aware that cognitive function tests may have differing neuro-molecular associations. Our results for MCP-1 were not consistent with previous findings (D. Galimberti et al., 2006), however the Galimberti study demonstrated increased levels of MCP-1 was increased in early Alzheimer’s (AD) pathology and then decreased with AD progression. IL-10 an anti-inflammatory cytokine acts to diminish pro-inflammatory cytokines (K. W. Moore et al., 2001). To our knowledge this was the first study too demonstrate a connection between inflammatory biomarkers and behavioural data. We could conclude from these studies that early cognitive neuropsychological testing is beneficial but the type of Stroop test used needs to be considered if treating neuroinflammation with anti-inflammatories.

Physical activity is known to reduce a number of comorbidities in T2DM. Lifestyle sedentariness is a major contributor to obesity and T2DM in western society. This study was established to observe the effect of acute single bout endurance testing (ET) and high intensity interval training (HIIT) on the inflammatory cytokines IL-6, IL-10, MCP-1, acute phase reactant CRP and IGF-1. ET and HIIT were conducted at least 7 days apart and blood was collected as per the exercise protocols. Results showed ET had an acute and prolonged inflammatory response with a significant decrease at 30 minutes post exercise with IL-6/IL-10 ratio (p =0.047) and a decrease of MCP-1 (p =
No significant changes were observed in CRP, IL-1β, IL-6, IL-10 and IGF-1.

MCP-1 has been associated with a reduction in the development of atherosclerosis, MetS and T2DM through a reduction in visceral fat reserves and following moderate levels of exercise in heart failure patients (Adamopoulos et al., 2001; Boring et al., 1998). Our findings of ET and HIIT showed MCP-1 was significantly reduced in both exercise regimes. This is contrary to findings where levels increased immediately after exercise (Zwetsloot et al., 2014), however this could be dependent upon flush out rates, or a long term decline in oxidative stress. A benefit in maintaining a constant reduction in MCP-1 is the decreased risk in T2DM and CVD (Deshmane et al., 2009). A similar mechanism could also explain why IL-10 was not significant (Markovitch et al., 2008). IL-6 levels were not significant and could partially explain why as the main inducer of CRP transcription CRP was not elevated. However IL-6 has an anti-inflammatory action and may negatively regulate the acute phase response (Ropelle et al., 2010). Maximal expression of CRP transcription occurs when both IL-6 and IL-10 are active (Ganter, Arcone, Toniatti, Morrone, & Ciliberto, 1989b). As seen in the previous Stroop paper (paper 9) the ratio IL-6/IL-10 was significant indicating the ratio may reflect a similar pathophysiological process, however IL-6/IL-10 did not provide a significant result when comparing the low and high quintile groups (paper 4). Further studies to clarify this relationship is required as an emphasis on multiple pathophysiology may be evident.
Final comment

This study has provided definitive answers by introducing new concepts in OS, inflammatory and coagulation biomarker studies relevant to T2DM and CVD and associated hyperglycaemic states including MetS, IFG, IGT and comorbidities. A novel and important finding was our ability to replicate controlled experimental results to hyperglycaemic patients in outpatient screening clinics. This has significant implications for transitioning our research into clinical use as a tool for the assessment of T2DM and CVD progression. More study is required on larger numbers to confirm our findings with some already underway at our laboratory. For example, we are investigating how Hcy responds to different combinations of MetS factors present. We are expanding our investigations into inflammatory biomarker ratios with a larger cohort to obtain a better understanding of what interactions are occurring in hyperglycaemia progressing to T2DM and CVD. We have demonstrated that the oxidative stress biomarker GSH:GSSG ratio in conjunction with appropriate inflammatory biomarkers can potentially be utilised as a more sensitive predictor in hyperglycaemia progression, as indeed a number of interleukins and interleukin ratios show promise subject to further confirmatory studies. These are and will be followed through with a larger population cohort. Our work presented in papers 5 and 6 relating to Hcy, GSH and 8-OHdG in MetS and medication usage respectively indicate our need to provide further research into emerging biomarkers in MetS which does include medication usage – in effect a non discriminatory community based screening study.
Much more work is required if we are to generate a suitable biomarker derived algorithm which will allow risk prediction for T2DM and CVD, however this thesis has provided more insight into the pathophysiology of hyperglycaemia without solving this very topical clinical dilemma.


American Diabetes, A. (2012). Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care, 35*(Supplement 1), S64-S71. doi:10.2337/dc12-s064


Bulhoes, K., & Araujo, L. (2007). Metabolic syndrome in hypertensive patients: correlation between anthropometric data and laboratory findings. Diabetes Care, 30(6), 1624-1626. doi:10.2337/dc06-2236


216


Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of


Hajer, G. R., van der Graaf, Y., Olijhoek, J. K., Verhaar, M. C., & Visseren, F. L. (2007). Levels of homocysteine are increased in metabolic syndrome patients but are not associated with an increased cardiovascular risk, in contrast to patients without the metabolic syndrome. *Heart, 93*(2), 216-220. doi:10.1136/hrt.2006.093971

Hajeri, G. R., van der Graaf, Y., Olijhoek, J. K., Verhaar, M. C., & Visseren, F. L. (2007). Levels of homocysteine are increased in metabolic syndrome patients but are not associated with an increased cardiovascular risk, in contrast to patients without the metabolic syndrome. *Heart, 93*(2), 216-220. doi:10.1136/hrt.2006.093971

Hajeri, G. R., van der Graaf, Y., Olijhoek, J. K., Verhaar, M. C., & Visseren, F. L. (2007). Levels of homocysteine are increased in metabolic syndrome patients but are not associated with an increased cardiovascular risk, in contrast to patients without the metabolic syndrome. *Heart, 93*(2), 216-220. doi:10.1136/hrt.2006.093971

Hajeri, G. R., van der Graaf, Y., Olijhoek, J. K., Verhaar, M. C., & Visseren, F. L. (2007). Levels of homocysteine are increased in metabolic syndrome patients but are not associated with an increased cardiovascular risk, in contrast to patients without the metabolic syndrome. *Heart, 93*(2), 216-220. doi:10.1136/hrt.2006.093971


stratification: A clinical study. *Wound Medicine, 8*, 24-30. doi:[http://dx.doi.org/10.1016/j.wndm.2015.03.003](http://dx.doi.org/10.1016/j.wndm.2015.03.003)


Kikuchi, H., Nanri, A., Hori, A., Sato, M., Kawai, K., Kasai, H., & Mizoue, T. (2013). Lower serum levels of total cholesterol are associated with higher urinary
levels of 8-hydroxydeoxyguanosine. *Nutr & Metab, 10*(1), 1-5. doi:10.1186/1743-7075-10-59


Patanella, A. K., Zinno, M., Quaranta, D., Nociti, V., Frisullo, D., Gainotti, and al. (2009). Correlations between peripheral blood mononuclear cell production of BDNF, TNF-alpha, IL-6, IL-10 and cognitive performances in...


http://www.nature.com/ni/journal/v14/n8/abs/ni.2639.html#supplementary-information


237


healthy adults are associated with reduced beta-cell function, increased intramyocellular lipid, and enhanced fat utilization during fasting. *J Clin Endocrinol Metab.*, 99(6), 2198-2207. doi:10.1210/jc.2013-4542


Events: A Prospective Study in the Elderly at Risk. *Arterioscler Thromb Vasc Biol*, 31(10), 2338-2344. doi:10.1161/atvbaha.111.231795


Appendices
Appendix A: Whole blood viscosity determination in diabetes management: perspectives in practice

Whole blood viscosity determination in diabetes management: perspective in practice

Ezekiel Uba Nwose, Eugene Butkowski, Nathan Cann
Institute of Clinical Pathology and Medical Research, South West Pathology Service
Smollett Albury NSW, Australia.

Background: Antiplatelet and antioxidant nutritional therapies (ANT) are commonly used in diabetes management. Guidelines recommend identifying deficiencies of antioxidant vitamins and condition of no contra indication for nutritional and antiplatelet respectively. **Aim:** To determine whether the guidelines recommendations for diabetes patients to be assessed for (1) antioxidant vitamins’ deficiencies and/or (2) whole blood viscosity (WBV) as indication of no antiplatelet contraindication. **Materials and Method:** Laboratory records were audited. 10,342 de-identified glycaemic index (HbA1c) requests received in 2008 were sorted into three groups based on level of control. (1) Poor (n = 1962, HbA1c > 8.1); (2) Good (n = 5616, HbA1c = 6.0 - 8.0) and (3) Excellent (n = 2764, HbA1c ≤ 5.9). All 57 cases with haematocrit and total protein results in the poor (n=30) and excellent (n=27) groups were selected for calculation and comparison of WBV levels. **Results:** None of the two guidelines’ recommendations are being followed as no case was requested for any antioxidant vitamin or WBV. Assessments of the latter show that WBV is statistically significantly lower (p < 0.05) in the group with excellent glycaemic control compared to the group with poor glycaemic control. **Conclusion:** Aspirin is one of the therapies in diabetes management. Its effect is modulated by WBV. ANT is alternative to aspirin and influences WBV. For patients that have full blood count and plasma protein results, WBV can be extrapolated at no extra cost to the health system. There is a need to raise awareness for the recommended guidelines for laboratory monitoring to be followed. (Nwose EU, Butkowski E, Cann N. North Am J Med Sci 2009; 1: 110-113).

**Keywords:** Antiplatelet therapy, antioxidant nutrition, diabetes mellitus, laboratory monitoring, whole blood viscosity.

**Correspondence to:** Dr. Ezekiel Uba Nwose, PhD, CSci, FIBMS, MAIMS. Institute of Clinical Pathology and Medical Research (ICPMR), South West Pathology Service, 590 Smollett Albury NSW 2640, Australia. Tel.: +61 260581651, Fax: +61 260581680. Email: ezekiel.nwose@gsahs.health.nsw.gov.au

245
Introduction

Observational studies have consistently supported the rationale for dietary therapy in diabetes mellitus [1]. This is shown in the evidence-based practice for Dietitians [2]. Antioxidants including glutathione (GSH), vitamin C and vitamin E amongst others have been identified as nutritional ingredients necessary for DM management [3]. The risk of harm from pro-oxidant radical forms of antioxidant vitamins is not in doubt. Hence, the Nutritionist’s protocol includes assessment, intervention and follow-up for a person with diabetes [2].

The following points about micronutrients are also acknowledged on the guidelines viz:

- Diabetes is synonymous to a state of oxidative stress.
- Optimal amount of antioxidants from nutrition benefits some of diabetes sufferers.
- If deficiencies of antioxidant vitamins are identified, supplementation can be beneficial.
- There is potential toxicity associated with excessive amounts of antioxidant vitamin supplements and is not recommended for diabetes patients if there is no underlying deficiency.
- Routine supplementation of the diet with antioxidants is generally not recommended because of the prevailing controversies.

By inference, nutritional management of diabetes is hinged on oxidative stress and the guideline for discrete prescription of antioxidant supplement is recommended to be based on evidence of deficiencies. That is, the laboratory testing of antioxidant levels.

It is known that vitamin C in the blood is associated with lower endothelial dysfunction and inversely related to fibrinogen concentrations and blood viscosity [4]. This is due to the capacity of vitamin C in maintaining the levels of erythrocyte GSH and vitamin E; and in effect attenuates erythrocyte oxidative stress induced hyperviscosity. It is also known that hyperviscosity in diabetes is strongly influenced by the excellence of glycaemic control [5-7]. Therefore, it is possible that dietary management of diabetes in clinical practice would necessarily involve assessment of vitamins C and E levels as well as whole blood viscosity (WBV).

This article addresses a practical question on evidence-based nutrition practice and anti-platelet therapy guidelines for diabetes management viz: how are discrete choices and the outcomes of antioxidant vitamins’ nutrition and/or contraindication for anti-platelet therapy determined in clinical practice? Given the provisions in the guidelines and the available in vitro diagnostic tests for antioxidant vitamins C and E, as well as WBV in clinical practice, the objective of this work was to investigate how many diabetes patients have been tested and followed up for these antioxidants and WBV.

Materials and methods

Ethical considerations and samples: This evaluation of de-identified data was approved by the HREC of South West Pathology Albury. The Pathology Service Albury receives samples from the Albury Base Hospital and clinics from Albury-Wodonga communities. Samples were collected by either nursing staff or medical officers. In some occasions, out-patients come into the laboratory where dedicated phlebotomists collect the blood samples. Samples for vitamins C and E are referred
to and tested at the Royal Prince Alfred laboratory Sydney, while WBV could be done on-sight.

It is assumed in this study that a number of patients would consult with dieticians, and that many would be medicating with aspirin. As participants in this study were de-identified and the outcome of this study provides for no direct or immediate personal clinical benefit to be offered, contact with patients was not made.

Data was acquired by downloading 2008 archived results, from the Auslab Laboratory Information System (LIS). All results of blood samples that were tested for glycaemic index (HBA1c) for the period of 1st January to 31st December 2008 were included. 10,342 results were pooled and sorted into three glycaemic control categories on the basis of decisive interpretation. (1) Poor (n = 1962, HbA1c > 8.1); (2) Good (n = 5616, HbA1c = 6.0 - 8.0) and (3) Excellent (n = 2764, HbA1c ≤ 5.9).

In this study, HBA1c was discretionally used as selection criteria to identify the otherwise de-identified diabetes subjects who are undergoing management and monitoring. Blood glucose level was part of information used in decisive interpretation of the result based on which sorting into groups has been done.

Laboratory records for each case were audited to identify any test for vitamins C, vitamin E and/or WBV. The tabulated results from this patient audit when possible included additional tests results for haematocrit and serum proteins, which were used to determine level of WBV according to the method of Tamariz and his group [8]. A total of 102 cases have results for haematocrit and plasma proteins, of which 57 cases comprising excellent group (n = 30) and poor group (n = 27) were selected for comparison of WBV levels. Statistical analysis was performed using ‘Student’s t-test assuming equal variances’. The hypothesis in the comparison was that hyperviscosity as a physiological manifestation of diabetes-induced oxidative damage would be less in the excellent group than in the poor group.

**Results**

None of the cases tested for glycaemic controls in 2008 were tested for any antioxidant vitamin deficiency or WBV. This is evidence of non-observance of the recommended guidelines.

Calculated whole blood viscosity seems close or similar in the two groups. However, analysis of the inter-quartile range shows it is statistically significantly lower (p < 0.05) in the group with excellent glycaemic control compared to the group with poor glycaemic control (Table 1).

**Table 1** Relative values in the two glycaemic control groups

<table>
<thead>
<tr>
<th></th>
<th>Poor</th>
<th>Excellent</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td>Mean</td>
<td>11.93</td>
<td>11.50</td>
</tr>
<tr>
<td>Median</td>
<td>11.95</td>
<td>11.43</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.37</td>
<td>1.30</td>
</tr>
</tbody>
</table>

**Discussion**

There is evidence that antioxidant vitamins are not being assessed in clinical practice as part of diabetes management. Two probable reasons may be responsible for this observation. Based on a clause in the guidelines that identification is difficult to ascertain [2], it is likely that practitioners
are unaware that the laboratory tests are available in clinical practice. This calls for awareness to be raised.

The impression (that identification of deficiencies of antioxidant vitamin level is difficult to ascertain) needs to be corrected, because validated methods for *in vitro* diagnostic use exist for vitamins C and E. In Australia, the tests can be bulk billed by public laboratories, which means the government has provided for it as service to citizens.

The second possible reason for non-observance of the guidelines' recommendations may be the controversy surrounding antioxidant efficacy. However, this reason in the face of increasing awareness for antioxidant nutrition and over-the-counter antioxidant supplements is unhelpful to the patients. This is especially given the acknowledged fact of antioxidant toxicities.

The same concern follows antiplatelet therapies such as aspirin. It was also observed that none of the participants were requested for WBV tests. A major reason may be the fact that the results from scientific research are quickly and erroneously categorized as relevant or irrelevant. Regrettably, reports on WBV fall into the latter category and attempts to discuss WBV most often turns out a waste of time [9].

WBV was assessed in this study, in patients for whom the haematocrit and plasma proteins were performed for different reason. The result shows that people with poorly controlled glycaemic index have statistically significantly (p < 0.05) higher level of viscosity compared to those that are excellently controlled (Table 1). This is expected as those with poor control would be experiencing more hyperglycaemia-induced oxidative stress with consequential reduced erythrocyte deformability and increased WBV [5-7].

This report provides further evidence that WBV is worse in poorly controlled diabetes. It further demonstrates that extrapolation of WBV level from haematocrit and plasma protein values is a valuable alternative method. A major insight to perspective in practice is that WBV can be determined for patients that have full blood count and plasma protein results, but at no extra cost to the health system.

It is known that WBV attenuates the efficacy of antiplatelet agents [10]. The significance is that individuals with low WBV would not benefit from antiplatelet agents. Therefore, until blood rheology problems are duly recognised and monitored in clinical practice, those who suffer from diabetes, but have low WBV will continue to be disadvantaged with antiplatelet therapy. Given that guidelines recommend identifying deficiencies of antioxidant vitamins and condition of no contraindication for nutritional and antiplatelet respectively; and given the result presented in this report; the question is: what is there to lose in laboratory monitoring of WBV in clinical practice? This audit did not seek to establish the reference value of WBV in the diabetes and healthy population. Beside the method adopted in this study, different methods exist for the determination of WBV and associated with this is different normal values. Perhaps, what needs to be established is what should be regarded as a reference values. This is especially important given the closeness in central values for the poorly and excellently controlled groups.
Conclusions

Clinical algorithms have been suggested to guide the use of dietary therapy using laboratory measures [3, 11]. However, the suggestions neither include the identifiable antioxidants, nor the index of effective pathophysiological haemorheology. Thus, the crucial factor of oxidative stress being managed by antioxidant nutrition is being overlooked. Furthermore, it is known that anticoagulants are not safe because of associated bleeding complications and treatment with antiplatelet as a substitute does not offer better safety. Aspirin is still one of the main therapies in diabetes management and its effect is modulated by WBV, which in turn is influenced by antioxidant nutritional therapy. Utilizing the available laboratory tests for antioxidant vitamins status in patients during dietician’s assessment would be keeping with guidelines. Determining WBV levels would be invaluable to optimize antioxidant nutrition outcomes, in addition to keeping with the guidelines for antiplatelet therapy.

Acknowledgement

This study was done with material and moral support of the management of the South West Pathology Service, Albury. The intellectual advice of the Medical Director, Dr Lance Meng is also appreciated.

References


Appendix B: Position paper for health authorities: archived clinical pathology data-treasure to revalue and appropriate


Western Pathology Cluster¹ — NSW Health, South West Pathology Service, 590 Smollett Albury and School of Community Health², Charles Sturt University Albury NSW 2640, Australia
Position paper for Health authorities: Archived clinical pathology data treasure to revalue and appropriate

E.U.Nwose1 RS Richards2, E Butkowski1 and Nathan Cann

Western Pathology Cluster1 – NSW Health, South West Pathology, 590 Smollett Albury and School of Community Health2, Charles Sturt University Albury NSW 2640, Australia

Summary

Archived clinical pathology data (ACPD) is recognized as useful for research. Given our privileged de-identified ACPD from South West Pathology Service (SWPS), attempt is made to estimate what it would cost any researcher without such privilege to generate the same data. The Ethics Committee of the Area Health Service approved a request for Dr. Uba Nwose to use de-identified ACPD acquired by the SWPS for clinical laboratory-based translational biomedical science research. 10-years (1999 - 2008) have been pooled to constitute the database. Data include blood sugar, cholesterol, D-dimer, ESR, glucose tolerance, haematocrit, HbAlc, homocysteine, serum creatinine, total protein and vitamins [C & E] amongst others. For this report, the bulk-billed-cost of tests were estimated based on number and unit price of each test performed. AU$17,507,136.85 is the cost paid by Medicare in the period. This amount is a conservative estimate that could be spent to generate such 10-years data in the absence of ACPD. The health/pathology service has not given any financial research grant. However, the support-in-kind is worth more than celebrated competitive research grants. It calls for revaluation by academic, research and scientific institutions the use of ACPD. For the countries whom such provision is non-existent, this report provides a ‘Position Paper’ to present to the directorates or institutes of health authorities to appropriate the value of ACPD and approve of their use as a research treasure and resource management tool.

Keywords: Archives, clinical laboratory, health authorities, research, resource management

Resume

L’archive des donnees cliniques pathologiques (ADCP) est reconnu comme utile en recherche, Etant donne que nos privil`eges du service pathologique du Sud Ouest (SWPS). Le comite ethique du service de sante etait Correspodance: Dr. E.U. Nwose, South West Pathology Service, 590 Smollett St., Albury 2640 NSW, Australie. E-mail: ezekiel.nwose@gsahs.health.nsw.gov.au; nwoseeu@hotmail.com approuve et utilise pour identifier et pour extraire les donnees cliniques pathologiques et les donnees des tests de laboratoires. Cette etude retrospective de 10-ans (1999 - 2008) servait comme la base de donnees. Les donnees collectees incluent le taux de glucose sanguin, taux de cholesterol, D-dimere, ESR, tolerance en glucose, hematocrite, HbAlc, homocysteine, taux de serum en creatinine, taux des proteines totales et les vitamines [C & E] parmi d’autres. Dans cette etude, le cout total des tests etait estimes en fonction du nombre et du prix unitaire de chaque test fait. AU$17, 507,136.85 est le cout des soins medicaux en cette periode. Cette somme est une estimative conservatrice qui pourrait depenser pour generer des donnees de 10 ans en absence de l’ADCP. Le service pathologique en sante n’a jamais donne une assistance financiere en recherche. Cependant, Un support moral est valeurux plus que de celebrer la competition financiere en recherche. Une reevaluation est necessaire par les academiciens, les chercheurs et les institutions scientifiques surf usage d’ADCP. Pour les pays ou de telle provision n’existe pas, Cette etude apporte une position a presenter aux autorites et
institutions en sante sur la valeur de l'ADCP et
d'approuver leur usage comme une banque des
donnees et ressources.

Introduction

Research funding or grant is a major factor that predetermines research activities. This factor can determine whether a potential Academic and/or researcher is engaged in service [1]. Archived clinical pathology data (ACPD) is recognized as useful for research. Hence the National Health and Medical Research Council (NHMRC) of Australia provides for the use of ACPD in its guidelines.

Databank and health informatics are well recognised resources in clinical research internationally. However, whether the concepts are appropriately valued by the owners and/or benefactors is something that could be discerned from a cursory evaluation of what the academic and research institutions celebrates more between the alternatives of (a) competitive research grant, or (b) research support-in-kind with ACPD.

Table 1: Number of tests (N) for different components of data.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>D-dimer</td>
<td>13.80</td>
<td>27.2</td>
<td>789</td>
<td>1.086</td>
<td>902</td>
<td>708</td>
<td>1.074</td>
<td>1.274</td>
<td>1.500</td>
<td>1.937</td>
</tr>
<tr>
<td>HCY</td>
<td>25.10</td>
<td>27.104</td>
<td>104</td>
<td>188</td>
<td>229</td>
<td>251</td>
<td>308</td>
<td>313</td>
<td>207</td>
<td>221</td>
</tr>
<tr>
<td>GOT</td>
<td>19.10</td>
<td>219</td>
<td>461</td>
<td>443</td>
<td>545</td>
<td>419</td>
<td>536</td>
<td>474</td>
<td>654</td>
<td>1.017</td>
</tr>
</tbody>
</table>

Vit. C& E: 31.15

0 | 6 | 12 | 8 | 11 | 7 | 4 | 7 | 14 | 15

Keys: Not all inclusive of components of database, Bulk-billing paid by Medicare on behalf of patients; *Vitamins E & E (charged together); BS: blood sugar (fasting or random); HCY: homocysteine; LP: lipid profile; GOT: oral glucose tolerance; S.Cr: serum creatinine

Table 2: Data monetary values (AUS$) from 1999 to 2008

<table>
<thead>
<tr>
<th>Test</th>
<th>1999</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>Total by test</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS</td>
<td>53.975</td>
<td>56.518</td>
<td>65.264</td>
<td>68.742</td>
<td>70.888</td>
<td>72.728</td>
<td>74.187</td>
<td>74.379</td>
<td>74.821</td>
<td>75.786</td>
<td>740.882</td>
</tr>
<tr>
<td>D-dimer</td>
<td>54.102</td>
<td>56.642</td>
<td>65.398</td>
<td>68.876</td>
<td>70.122</td>
<td>72.062</td>
<td>74.121</td>
<td>74.433</td>
<td>74.875</td>
<td>75.840</td>
<td>740.938</td>
</tr>
<tr>
<td>ESR</td>
<td>54.102</td>
<td>56.642</td>
<td>65.398</td>
<td>68.876</td>
<td>70.122</td>
<td>72.062</td>
<td>74.121</td>
<td>74.433</td>
<td>74.875</td>
<td>75.840</td>
<td>740.938</td>
</tr>
<tr>
<td>FBC</td>
<td>54.102</td>
<td>56.642</td>
<td>65.398</td>
<td>68.876</td>
<td>70.122</td>
<td>72.062</td>
<td>74.121</td>
<td>74.433</td>
<td>74.875</td>
<td>75.840</td>
<td>740.938</td>
</tr>
<tr>
<td>HbAlc</td>
<td>54.102</td>
<td>56.642</td>
<td>65.398</td>
<td>68.876</td>
<td>70.122</td>
<td>72.062</td>
<td>74.121</td>
<td>74.433</td>
<td>74.875</td>
<td>75.840</td>
<td>740.938</td>
</tr>
<tr>
<td>HCY</td>
<td>54.102</td>
<td>56.642</td>
<td>65.398</td>
<td>68.876</td>
<td>70.122</td>
<td>72.062</td>
<td>74.121</td>
<td>74.433</td>
<td>74.875</td>
<td>75.840</td>
<td>740.938</td>
</tr>
<tr>
<td>LP</td>
<td>54.102</td>
<td>56.642</td>
<td>65.398</td>
<td>68.876</td>
<td>70.122</td>
<td>72.062</td>
<td>74.121</td>
<td>74.433</td>
<td>74.875</td>
<td>75.840</td>
<td>740.938</td>
</tr>
<tr>
<td>GOT</td>
<td>54.102</td>
<td>56.642</td>
<td>65.398</td>
<td>68.876</td>
<td>70.122</td>
<td>72.062</td>
<td>74.121</td>
<td>74.433</td>
<td>74.875</td>
<td>75.840</td>
<td>740.938</td>
</tr>
<tr>
<td>S. Cr</td>
<td>54.102</td>
<td>56.642</td>
<td>65.398</td>
<td>68.876</td>
<td>70.122</td>
<td>72.062</td>
<td>74.121</td>
<td>74.433</td>
<td>74.875</td>
<td>75.840</td>
<td>740.938</td>
</tr>
<tr>
<td>Sr. Protein</td>
<td>54.102</td>
<td>56.642</td>
<td>65.398</td>
<td>68.876</td>
<td>70.122</td>
<td>72.062</td>
<td>74.121</td>
<td>74.433</td>
<td>74.875</td>
<td>75.840</td>
<td>740.938</td>
</tr>
<tr>
<td>Vit. C&amp; E</td>
<td>54.102</td>
<td>56.642</td>
<td>65.398</td>
<td>68.876</td>
<td>70.122</td>
<td>72.062</td>
<td>74.121</td>
<td>74.433</td>
<td>74.875</td>
<td>75.840</td>
<td>740.938</td>
</tr>
</tbody>
</table>

Keys: *Price x N* based on Table 1; *Vitamins E & E (charged together); BS: blood sugar (fasting or random); HCY: homocysteine; LP: lipid profile; GOT: oral glucose tolerance; S.Cr: serum creatinine
Objective

Given our privileged de-identified ACPD from South West Pathology Service, attempt is to estimate what it would cost any researcher, without such privilege, to generate the same data for some research purposes. The objective of this commentary is to perform valuation in monetary terms with a view to estimating the worth of the 10-year data privilege.

Methods

Based on a personal research initiative and proposal to the Human Research Ethics Committee (HREC) of the Greater Southern Area Health Service (GSAHS) and the Operational Manager of the South West Pathology Service, a request was granted to obtain de-identified ACPD acquired by the laboratory service for a consecutive period of 10-years (1999 - 2008). As at the time of preparing this report, some clinical chemistry and haematology results have been pooled. These include blood sugar, cholesterol, D-dimer, ESR, glucose tolerance, haematocrit, HbAlc, homocysteine, serum creatinine, total protein and vitamins [C & E] amongst others. The bulk-billed-cost was calculated based on number of each test performed during the periods. Health authorities in the paper refer to administrators in the directorates or institutes of health and pathology managers.

Findings

The number of tests (N) pooled for each test-parameter for each year from 1999 - 2008 is presented (Table 1). Included is the ‘Bulk-billing’ unit price for each test. Multiplying number of test with unit price, it was found that AU$17,507,136.85 is the cost paid by Medicare in the period of 1999 - 2008 for the test-parameters included in this valuation exercise (Table 2).

This amount is a conservative estimate that could be spent to generate such 10-years data in the absence of ACPD, because there are other tests such as C-reactive proteins, lipoprotein-A, and urinary iodine, amongst others. Further, most of the liver and renal function tests are in panels that incur higher costs. Therefore, more than AU$17,507,136.85 would be spent to generate such 10-years data in the absence of ACPD.

Discussions

From this study, the result shows that the GSAHS as a health authority, and in particular South West Pathology Service, has supported a research initiative with data valued at over seventeen and half million Australian dollars (AU$17,500,000.00) (Table 1). This is arguably incredible with the data valued in this study excluding bacteriology data and not inclusive of all biochemistry undertaken on each episode.

However, value analysis of reports from similar database lends credence to the fact that ACPD is worth more than may be imagined. There is a report on 3- years blood culture results audit in which 5216 sets of specimens were pooled [2]. Based on Medicare bulk- bill fee of AU$30.95 per blood culture, the support-in-kind for that study is worth AU$161,435.20. The same valuation of support-in-kind can be applied to our other related laboratory based studies [3-5], and so many published and ongoing laboratory based prevalence and/or retrospective studies [6-8].

What this report brings to the fore is that the real worth of such supports by the health management is not usually valued. The obvious effects include the fact that those who have such privilege may not appreciate it; and the scientific communities may not celebrate the academic Staff with such privileged database like when another academic wins a competitive research grant. Further, the health authority may be literally funding prospective research for which ACPD could achieve if gainfully employed in a retrospective study.

Although, the objective was to estimate what it would cost any researcher without the privilege of ACPD to generate an equal amount of data, there are several other known advantages to glean. These include the following points

1. Demonstrate the strength to go into international collaborative research for cardiovascular risk assessment in prediabetes studies (CRAPS), which we hypothesized [9], This is similar to the agenda of Downing and his group [10].
2. Propagate the feasibility of using retrospective data for a longitudinal
analysis in CRAPS. In consideration of a model for the assessment of diabetic macrovascular complications in prediabetes, it is necessary to have a data set obtained during the prediabetes phase. These would be followed with another data set obtained from those individuals who progress to DM-CVD co-morbidity. It would be feasible when such data sets are obtained from ACPD audit. Importantly, it would be more accurate relative to prospective research protocols that involve biased discretionary adjustment and/or control for confounding factors and consideration of participant compliance issues.

3. Demonstrate value of ACPD for clinical laboratory employees who are interested in research. Two of the factors responsible for job dissatisfaction among new medical scientist are poor support for postgraduate studies, and lack of recognition for ‘research & development’ opportunities. Addressing these factors would mean that clinical research need to be encouraged more than it is currently. That is, support the hospital scientists to do research while still rendering diagnostic services in order to encourage postgraduate studies and/or reduce the sense of lack of recognition [11].

Interestingly, given the issue of enabling current scientist to gain the qualifications that will allow them to fill the senior positions in the next decade [12], this has become apparently necessary.

Recommendations for academic and research institutions

The 2020 Vision of the Manager of the 21st Century indicates that executives will measure performance on outputs rather than inputs (Peta McGrath: Ensure Team Effective workshop 2010). Therefore, managers of academic and research institutions would necessarily need to review how they celebrate or value the alternatives of (a) competitive research grant, and (b) research support-in-kind with ACPD. Obviously, the review has to put more emphasis on output; and it may not be in dispute that this has been the case. However, this report brings out the following two emphases

1. It draws the attention of academic institutions to review how their lecturers who have affiliations with clinical pathologies appropriate and treasure such privileges. It would benefit from such a review to determine whether the universities had acknowledged such opportunities as they did competitive research grants. The determination will inform whether there has been sufficient emphasis and/or incentive for the lecturers to appreciate and appropriate ACPD as a treasure.

2. Foster the development of university teaching philosophies. University teaching now involve the concept and tenets of clinical reasoning and experiential learning as well as teaching-and-research nexus [13, 14]. ACPD provides a base framework that enhances university-pathology partnership, which in turn enhances professional medical science undergraduates’ experiential teaching and engagement in research.

Position statement for health authorities

Perhaps, the foremost step is appreciation of ACPD as research treasure by laboratory administrators and managers whose laboratories are, or shall be, affiliated to any research organisation. Here, the issue is to consider ACPD as an asset for appropriate resource management.

This report calls for the attention of health/ pathology administrators and policy makers to appreciate and appropriate ACPD in their power as a matter of further laboratory productivity. That is, revalue ACPD with a view to consider redefining (e.g. in monetary terms) their research support as indication of conscious resource management. It is also an opportunity to demonstrate to medical scientists in diagnostic pathology that support is available for postgraduate studies, or research and development.

As academic-industry partnerships are developing, questions bordering on valuation of research are also becoming increasingly important. The need to develop methods for value assessment of academic research is equally becoming imperative [15]. For instance, what is the valuation protocol or standard of research performance or productivity for Clinicians and Medical Scientists in academics? Incidentally, this group of academics necessarily have to adopt the concepts or tenets of clinical reasoning, experiential learning and teaching-and-research nexus. Especially, when considering academic-and-health industry partnership with regards to development of new diagnostic test, it should be noteworthy that the difficulties that health authorities face include issues of accuracy, cost and relativity [16]. ACPD provides for
assessment of relativities that could benefit the health management.

Further advantage that can arise from nonlimitation and promotion of the use of ACPD for research is the development of evidence-based messages aimed at advising clinicians and other stakeholders on disease management and prevention. Examples on this include laboratory-based advice on blood culture result and perspectives in practice regarding whole blood viscosity [2, 4, 5].

Conclusion

We report a non-financial research grant that is worth millions of dollars, which is incredibly more than celebrated competitive research grants. This calls for better appreciation and appropriation of ACPD by the academic institution who require such data for research productivity, research medical scientists who have the privilege and the health authorities who have the power to offer such grants. The potential for CRAPS and other obvious advantages are briefly highlighted.

Acknowledgements

Receipt of an approval from the Greater Southern Area Health Service, NSW Health, through the HREC committee is gratefully appreciated. The work has been done partly with material support of the South West Pathology Service Albury Australia.

References

1. Paterlini M. When in Rome, reform. Radical reform of the Italian research and education systems is needed to address the lack of autonomy and lack of funding. EMBO Rep 2009; 10: 128-131
8. Sanders R. Mining the Swedish clinical archives to develop pharmacogenomic tests. Mol Diagn 1999; 4: 319-325
Appendix C: Serum uric acid and albumin levels and estimated glomerular filtration rate: oxidative stress considerations

Serum uric acid and albumin levels and estimated glomerular filtration rate: oxidative stress considerations

Phillip Bwititi, Uba Nwose, Sharon Nielsen, Henk Ruven, Wouter Kalle, Ross Richards and Eugene Butkowski

1 School of Biomedical Sciences, Charles Sturt University, New South Wales
2 ICPMR, Nepean Hospital Pathology, New South Wales
3 School of Computing and Mathematics, Charles Sturt University, New South Wales
4 Department of Clinical Chemistry, St Antonius Hospital, Netherlands.
5 School of Community Health, Charles Sturt University, New South Wales.
6 ICPMR, South West Pathology Services, New South Wales

Abstract

Uric acid has both antioxidant and oxidant properties, is excreted by the kidney and is associated with development of kidney disease. Albumin, another antioxidant is also excreted by the kidney in some renal diseases. The association and correlation of serum uric acid, albumin and estimated glomerular filtration rate (eGFR) could indicate the potential of renal function testing in evaluation of oxidative stress. This preliminary retrospective study looked at data on eGFR and serum levels of uric acid and albumin in 274 patients (120 males and 154 females) with an average age of 60.1 years but did not take into account any existing conditions or medication. Serum albumin levels were reduced as eGFR decreased and an inverse relationship between eGFR and serum uric acid levels with a statistically significant but weak partial correlation was also observed after accounting for sex and age ($r = -0.32$; $p < 0.001$). A controlled study on oxidant/anti-oxidant capacity of uric acid and other metabolites and relate these to renal function could be performed and this could lead to an indication of oxidative damage to the kidneys.

Keywords: GFR, eGFR, uric acid, albumin, oxidative stress, renal function
Introduction

Although there is information with regard to the presence of oxidative stress in various diseases, the mechanisms of this oxidative stress is yet to be fully elucidated or explained. Oxidative damage is reported for most diseases but clear correlation between most diseases and oxidative stress is lacking. This is perhaps due to little knowledge of the molecular mechanisms leading to oxidative stress and of the methods that measure oxidative stress (Giustarini et al. 2009).

Oxidative stress can contribute towards renal injury (Sarafidis et al. 2006; Sarafidis and Grekas 2007; Karamouzis et al. 2008) and reactive oxygen species are implicated in various diseases including renal disease (Cachofeiro et al. 2008). The levels of certain markers of oxidative stress increase with increasing degrees of renal dysfunction and it has been shown that reactive oxygen species increase in a graded manner as renal function deteriorates (Cachofeiro et al. 2008; Ferretti et al. 2008; Karamouzis et al. 2008). Inverse correlations between different markers of oxidative stress and glomerular filtration rate (GFR) have also been reported (Terawaki et al. 2004; Cachofeiro et al. 2008). For instance, Terawaki et al. (2004) using the redox state of human serum albumin as a marker of oxidative stress reported that oxidised albumin was increased before dialysis and had an inverse correlation with renal function.

Uric acid, a pro-oxidant and antioxidant, is excreted by the kidney and it is elevated in some cases of renal impairment. Durante et al. (2010) citing several studies, reported uric acid to be a natural and powerful antioxidant. At physiological concentrations, uric acid protects erythrocytes against peri-oxidative damage (Ames et al. 1981) and increases the activity of superoxide dismutase in the aortic wall of ApoE-/-mice (Hink 2002). It is also a scavenger of peroxynitrites (Hooper et al. 2000; Scott and Hooper 2001) which are oxidising agents that can interact with cell constituents inducing cell injury (Gow et al. 2004; Glantzounis et al. 2005). Patschan et al. (2007) and Durante et al. (2010) also noted that acute and mild elevation of uric acid could protect against ischaemic injury in mice. In addition to being a radical scavenger, uric acid can chelate metal ions such as iron and copper, converting them to poorly reactive forms that are unable to catalyse free radical reactions (Davies et al. 1986; Glantzounis et al. 2005).

Although hyperuricaemia has been proposed to be a major antioxidant, it has also been shown to be associated with the development of a number of diseases including kidney failure (Alderman et al. 1999; Johnson et al. 2003; Sautin and Johnson 2008). Therefore, there is also the potential for hyperuricaemia to be pro-oxidant. The pathogenesis of many diseases is not clear, but oxidative stress appears to be a common feature (Berg et al. 2004; Furukawa et al. 2004; Stocker and Keaney 2004; Wellen and Hotamisligil 2005; Houstis et al. 2006; Sautin and Johnson 2008) and with the ability of uric acid to be pro-oxidant under certain conditions, this creates the urate oxidant-antioxidant paradox (Sautin and Johnson 2008).

It is important to note that most antioxidants become pro-oxidants by default as a metabolic by-product of an antioxidation reaction. It is the process of regenerating an antioxidant from its pro-oxidant form by other antioxidants (e.g. vitamin C regenerates GSH from GSSG) that culminates in co-antioxidant interactions. Therefore, the ability of uric acid to become pro-oxidant may not counter its antioxidant property, but connotes the possible existence of regenerating co-antioxidant. The issue is that the concept of uric acid as a powerful antioxidant challenges the
traditional notion regarding the metabolite being a toxic waste product of metabolism. It is therefore important to understand the urate oxidant-antioxidant paradox.

On the subject of cardiovascular events, Johnson et al. (2003) noted that the beneficial actions of uric acid may counter its potential detrimental effects hence it may not be an independent risk factor for the disease. Such observations warrant the studying of the protective and/or toxic effects of uric acid in various diseases. Increases in uric acid in renal disease due to reduction in GFR and renal urate excretion (Johnson et al. 2003) could be beneficial in protection from oxidative stress. Whether hyperuricaemia is a biomarker for antioxidant capacity or oxidative stress status is an impasse to resolve for diagnostic utility. Hence it is important to investigate its association with stages of estimated glomerular filtration rate (eGFR).

Hypothesis

In the context of renal dysfunction and assuming absence of any confounding factor, low eGFR and hypoalbuminaemia as well as hyperuricaemia are implicated. We hypothesize that if hyperuricaemia in renal disease (due to reduction in GFR) is beneficial in protection from oxidative stress, then the increase in uric acid level would not have a significantly negative correlation with eGFR and serum albumin level, because of the metabolic conversion of uric acid in the process of antioxidation reaction. If this is not the case, hyperuricaemia could be just accumulation of waste product of metabolism and more of a toxic prooxidant than antioxidant.

Objective of study

In consideration of the report of Terawaki et al. (2004) on albumin’s inverse correlation with renal function in addition to our hypothesis, this retrospective pilot study investigated serum uric acid and albumin levels and eGFR with a view to establishing correlation among these routinely assessed clinical parameters. The presence of any existing disease and medication were not taken into account at this preliminary stage.

Materials and methods

Ethical considerations

This work is part of a clinical laboratory-based Biomedical Science Research supported materially by Albury South West Pathology, a unit of the Pathology West of NSW Health, Australia. The Ethics Committee of the Area Health Service approved the use of the de-identified data. All tests were performed at the Albury laboratory of South West Pathology.

Data

This preliminary retrospective study looked at data from South West Pathology, NSW for 2008 on eGFR, serum uric acid and albumin levels to investigate correlations among these parameters. This data involved 274 results of males (120) and females (154) and did not take into account any other disease or medication as this was sometimes not recorded. The ages ranged from 19 to 90 years and an average age of 60.1 years.

Serum albumin was measured as part of routine ‘liver function testing’. eGFR was estimated and generated automatically for qualifying patients who had renal function panels, comprising electrolytes, urea and creatinine performed. All tests were measured using the Dimension® RXL (Siemens) automated analyser. Estimated GFR was calculated according to the modified Diet in Renal Disease (MDRD) formula as follows:

\[ \text{Males} = 175 \times \left( \frac{\text{CREA}}{88.4} \right)^{1.209} \times \text{age}^{0.203} \]
\[ \times 1 \quad \text{Females} = 175 \times \left( \frac{\text{CREA}}{88.4} \right)^{1.209} \times \text{age}^{0.411} \times 0.742 \]

eGFR was reported (i) for patients from ages > 18 years; (ii) not reported if creatinine levels had risen > 70 pmol/L in previous seven days; and (iii) as > 60 mL/min if estimate was more than 60 mL/min.

Statistical analysis

Table 2: Average eGFR, age, serum albumin and uric acid levels at various eGFR ranges in males.

<table>
<thead>
<tr>
<th>eGFR (mL/min)</th>
<th>Age (years)</th>
<th>eGFR (mL/min)</th>
<th>Albumin (g/L)</th>
<th>Uric acid (mmol/L)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;30</td>
<td>72.1</td>
<td>17.6</td>
<td>33.7</td>
<td>0.419</td>
<td>7</td>
</tr>
<tr>
<td>31-40</td>
<td>72.1</td>
<td>35.7</td>
<td>37.4</td>
<td>0.431</td>
<td>7</td>
</tr>
<tr>
<td>41-50</td>
<td>69.9</td>
<td>44.6</td>
<td>34.3</td>
<td>0.508</td>
<td>8</td>
</tr>
<tr>
<td>51-60</td>
<td>73.9</td>
<td>56.8</td>
<td>37.3</td>
<td>0.38</td>
<td>11</td>
</tr>
<tr>
<td>61-70</td>
<td>64.8</td>
<td>66</td>
<td>40.6</td>
<td>0.374</td>
<td>17</td>
</tr>
<tr>
<td>71-80</td>
<td>60.1</td>
<td>74.6</td>
<td>38.6</td>
<td>0.359</td>
<td>16</td>
</tr>
<tr>
<td>81-90</td>
<td>58.4</td>
<td>87.8</td>
<td>39.1</td>
<td>0.345</td>
<td>53</td>
</tr>
</tbody>
</table>

*Raw data, which were transformed for partial correlation analysis

\[ \text{Males} = 175 \times \left( \frac{\text{CREA}}{88.4} \right)^{1.209} \times \text{age}^{0.203} \]
\[ \times 1 \quad \text{Females} = 175 \times \left( \frac{\text{CREA}}{88.4} \right)^{1.209} \times \text{age}^{0.411} \times 0.742 \]
The aim of the analysis was to define the strength of the relationship between eGFR, albumin and uric acid. This was achieved using a two-step procedure in which the partial correlations between these response variables were calculated. As the data resulted from an observational study where the results were likely to be affected by age and gender among other variables, an analysis of covariance was used to account for these effects. The residuals that were obtained from these models were then subjected to a partial correlation analysis.

A different analysis of covariance model was required for each variable to ensure that the residuals conformed to the usual assumptions of normality, independence and constant variance. The model for eGFR included the main effect of gender, terms for a second order polynomial regression on age and their interactions. A power transformation was used on the eGFR result to ensure the model assumptions were met. Albumin and uric acid were also transformed using power transformations however a simpler model with the main effect of gender, the simple linear regression on age and the interaction of these two terms was used.

Results

Tables 1-3 show a decrease in serum albumin levels as eGFR goes down and at the same time an inverse relationship is observed between eGFR and serum uric acid levels and eGFR and age. This relationship is seen in both sexes.

The simple correlation (Pearson's correlation coefficient) was initially calculated using the raw data. There was a weak positive relationship between eGFR and serum albumin levels ($r = 0.11$), and this correlation was not statistically different from zero ($p = 0.698$). The correlation between serum uric acid and serum albumin levels was essentially equal to zero ($p = 0.963$). eGFR and serum uric acid levels had a stronger negative correlation ($r = -0.363$). This correlation was the only simple correlation that was significantly different to zero ($p < 0.001$).

The partial correlation analysis confirmed the results from the simple correlation tests (Table 4). After accounting for sex and age, eGFR and serum albumin levels had a partial correlation of ($r = 0.0690$). This partial correlation was not statistically different from zero ($p = 0.257$). The partial correlation between eGFR and serum uric acid, after accounting for sex and age, was statistically different to zero ($r = -0.32; p < 0.001$). After accounting for sex and age the correlation between serum albumin and uric acid was not significant different from zero ($r = -0.07; p = 0.246$).

Discussion

The inverse relationship between GFR and age observed (Tables 1-3) is not surprising as studies have shown that GFR declines with advancing age. USA and Finnish studies have shown that GFR progressively increases up to 18 years and declines thereafter (Wahl et al. 2003). Berg (2006) found significant decline in absolute and relative GFR with age in males as early
as 20 years, but not in females. Wesson (1969) cited in Berg (2006), reported delayed and slower fall in GFR with age in women. Some authors have however stated that GFR is about the same in males and females when GFR is related to body surface area (Rule et al 2004; Grewal and Blake 2005) and there appeared to be no differences in GFR between the sexes in this study.

Serum uric acid levels progressively increased as eGFR decreased (Tables 1-3) in both sexes in this study. Furthermore, eGFR and serum uric acid levels had negative correlation after accounting for sex and age. This observation is possibly a demonstration of the lack of evidence that the un-excreted uric acid is being used as antioxidant perhaps protecting against a decline in GFR. Therefore, we uphold the traditional opinion that hyperuricaemia is the accumulation of waste product of metabolism and infer that it may be more of a toxic waste (including pro-oxidant potentials) than antioxidant.

Miyatake et al (2011) found in Japanese men without medications and after one year of life-style changes (including balanced nutrition and exercise), a negative correlation between changes in eGFR and changes in serum uric acid levels, and concluded that reducing uric acid levels could be useful for improving eGFR. Using longitudinal analysis, Yen et al (2009) showed that serum uric acid levels were associated with eGFR and decline in renal function in elderly Taiwanese subjects. The authors reported that asymptomatic hyperuricaemia was observed in chronic kidney disease, possibly a reflection of decreased renal uric acid excretion and this could also be responsible for pathogenic progression of chronic kidney disease through various mechanisms.

The mechanisms underlying the association between high values of uric acid and diminishing renal function would benefit from further elucidation. Serum uric acid can function as a pro-inflammatory molecule with capacity to act as a pro-oxidant and as an antioxidant as well (Johnson et al 2003; Rosolowsky 2008). Increased serum uric acid level may augment the risk for development of renal disease. For example in a community-based study of Japanese adults, hyperuricaemia emerged as the only significant risk factor of renal failure besides age, and it was reported to be strongly more predictive than proteinuria (Iseki et al 2001; Rosolowsky et al 2008). Elevated uric acid levels can cause endothelial dysfunction through stimulation of vascular smooth muscle proliferation resulting in thickening of afferent arterioles of glomerulus, and hyperuricaemia inhibits the release of nitric oxide within the vasculature of the kidneys hence reducing renal blood flow and GFR (Johnson et al 2003; Weaver et al 2005; Alasia et al 2010).

In this study we observed a decrease in serum albumin levels that loosely corresponded to a decline in eGFR (Tables 1-3). Decreased levels of serum albumin have been shown to predict mortality in patients with end stage kidney disease and in patients with acute renal failure (Chertow et al 1998; Obialo et al 1999; Owen 1993; Chawla et al 2005). In critically ill patients, decreased levels of serum albumin have often been ascribed to poor nutritional status but serum albumin level can also fall significantly in response to inflammation and capillary leakage (Moshage et al 1987; Chawla et al 2005). Hypoalbuminaemia is highly prevalent in kidney failure and is associated with an increased mortality risk (Foley et al 1996; Menon et al 2005); enhanced protein catabolism by pro-inflammatory cytokines in inflammation and nutritional status are possibly involved (Menon et al 2005). No correlation was observed between serum uric acid levels and serum albumin levels in this pilot study (Table 4). Given that renal dysfunction indicated by low eGFR may cause hyperuricaemia concomitant with hypoalbuminaemia, it is logical to expect that serum uric acid level would be inversely correlated with serum albumin concentration as it is associated with eGFR. Therefore, the observation of no correlation may be an apparent demonstration of complex pathophysiology and perhaps the influence of medication that the patients could have been taking. The study did not examine a particular disease, medication or population. However among Taiwanese adults with type 2 diabetes mellitus, Tseng (2005) reported that hyperuricaemia correlated with increased albumin excretion rate. A direct correlation between GFR and serum albumin levels in children with nephrotic syndrome has also been observed (Lowenborg and Berg 1999).

This study is cognisant of the fact that medication and types and duration of
diseases were not considered. The major aim was to see if a relationship existed between eGFR and serum uric acid and albumin levels. Further research is needed to particularise diseases in relation to serum uric acid and albumin levels. Urinary albumin needs to be measured with a view to separate kidney diseases presenting with albumin loss and those that do not. Use of Modification of diet in renal disease (MDRD) formula (Levey et al 1999) may underestimate rate decline in GFR (Lippi et al 2009) and this is pertinent in correlation studies and when relating GFR to age.

Conclusion

An inverse relation between eGFR and age was observed. Serum uric acid levels and eGFR showed an inverse relationship. Decreased serum albumin levels were not significantly associated with declining eGFR or serum uric acid levels. The observations may be an indication of the non-existence of significant metabolic process such as antioxidation to counteract the accumulation of toxic uric acid associated with renal disease. More studies need to be carried out on eGFR, uric acid and albumin levels focusing on the contributions of antioxidant and pro-oxidant properties of uric acid as well as albumin in oxidative stress, which is known to contribute to kidney disease.

Conflict of interest

None declared.

References


Menon V, Greene T, Wang X, Pereira AA, Marcovina SM,


Appendix D: Serum bilirubin and lipoprotein-a: how are these associated with whole blood viscosity?

Serum bilirubin and lipoprotein-a: How are these associated with whole blood viscosity?

E. U. Nwose12, R. S. Richards1, P. Bwititi3, E. Butkowski4

1School of Community Health, Charles Sturt University, Albury, NSW, Australia, Institute of Clinical Pathology & Medical Research, Nepean Hospital Pathology, Kingswood, NSW, Australia, 3School of Biomedical Sciences, Charles Sturt University, Wagga Wagga, NSW, Australia, Institute of Clinical Pathology & Medical Research, South West Pathology, Albury, NSW, Australia

Background: It has been demonstrated that oxidative stress can induce red blood cell rigidity and haemolysis, which in turn can cause hyperviscosity and hyperbilirubinaemia, respectively. However, haemolysis may be associated with a low level of haemoglobin, which reduces whole blood viscosity (WBV). Bilirubin can behave as antioxidant or oxidant, and one uncharted course for diagnostic pathology is how or whether bilirubinaemia and viscosity are associated. Further, oxidative stress is now being assessed using lipoprotein-a (Lp(a)), among other things but whether it is associated with blood viscosity has not been established.

Aim: This study investigates the association and correlation of haemoglobin level and WBV with serum Lp(a) and bilirubin levels in a general population of patients.

Materials and methods: Sixty-eight cases that were tested for Lp(a), concomitantly with full blood count and liver function, in our archived clinical pathology database were used in this study. WBV levels were determined using a validated formula. Multivariate and univariate analyses as well as correlation were performed.

Results: WBV was found to be significantly associated with bilirubin (P < 0.02), but not with Lp(a). Haemoglobin concentration was inversely correlated with Lp(a) (P < 0.04), but not with bilirubinaemia.

Conclusion: This pilot study suggests that hyperbilirubinaemia and hyperviscosity are associated and positively correlated. Consideration of whether serum bilirubin (as an indirect index of oxidative stress) can be used in combination with WBV (as index of macrovascular effect of oxidative stress) to assess oxidative damage is recommended.

Keywords: Clinical assessment, Diagnostic pathology, Haemolysis, Hyperbilirubinaemia, Hyperviscosity, Oxidative stress

Introduction

In clinical diagnostic practice, bilirubin measurement is an important component of the test panel for liver functions. Total bilirubin is commonly used for monitoring of patients with anaemia who are suspected of disorders associated with haemolysis. Hyperbilirubinaemia has been associated with circadian rhythm and oxidative stress.1 Circadian rhythm has been linked to several physiological processes including antioxidant activities versus oxidative stress and blood viscosity.2-4 Interestingly, and typical of paradoxical properties of antioxidants, bilirubin can behave as an antioxidant or oxidant depending on its concentration.5 Particularly, bilirubin at low concentration is an antioxidant in neonatal jaundice,6 but during haemolysis it is possible that bilirubin free radicals such as lumirubin may be generated;6,7 thereby conferring oxidant properties on bilirubin.

The concept of bilirubin being associated with oxidative stress is interesting with potential for its use in laboratory medicine diagnosis, because it is an established routine laboratory index. Given that oxidative stress may be associated with serum bilirubin level on one hand, and exacerbates whole blood viscosity (WBV) on the other, it is worth investigating whether bilirubinaemia could be associated with WBV.

Further, evidence of oxidative stress and a concomitant demonstration of any of the effective vascular events including increase in WBV is necessary to establish oxidative damage.8 If bilirubinaemia is associated with WBV; it would be worth investigating how or whether the two parameters are correlated, given the antioxidant and pro-oxidant properties of...
bilirubin. A determination of association and correlation could mean a possibility that the two parameters can be used to assess oxidative damage.

Lipoprotein(a) (Lp(a)) is a laboratory marker that is emerging as a possible risk factor for cardiovascular diseases (CVD). Studies have provided support to the hypothesis that Lp(a) attenuates fibrinolysis and promotes coagulation, thus affirming the notion that high-level plasma Lp(a) is a risk factor for CVD. Lipoprotein(a) (Lp(a)) is attributed to have a role as an acute phase inflammatory reactant and is associated with oxidative stress. It is believed that high plasma Lp(a) level does not translate to causality of CVD. Instead, it is thought to confer additional risk only in the presence of traditional factors. However, haemolysis, Lp(a) and oxidative stress constitute markers of lipoprotein metabolism. One of the problems in diagnostic pathology, is that Lp(a) measurement is in urgent need of standardization. Lp(a) is yet to be an established biomarker, and only very few clinicians request the test. It is the intention in this study to investigate how Lp(a) is related to anaemia, bilirubinaemia, and WBV.

The theory that oxidative stress is a major contributing factor to blood viscosity dates back more than four decades. An estimate of WBV can now be determined from haematocrit (HCT) and serum total proteins (TP) levels thus:

High shear rate: \( WBV(208 \text{ seconds}^{-1}) = (0.12 \times \text{HCT}) + 0.17(\text{TP} - 2.07) \)

Low shear rate: \( WBV(0.5 \text{ second}^{-1}) = (1.89 \times \text{HCT}) + 3.76(\text{TP} - 78.42) \)

where HCT = haematocrit (%) and TP = serum total proteins (g/l).

Low WBV, which is related to anaemia, complicates low shear rate and constitutes part of the abnormalities that are associated with a decreased vasodilating response to endothelial mechanical stimulation. However, the level of WBV increases with HCT and underpins the principle of treating hyperviscosity in patients with polycythemia. Conversely, anaemia if due to haemolysis is associated with high serum bilirubin level and low blood viscosity.

Hypotheses

Oxidative stress can induce red cell rigidity and haemolysis (Fig. 1), which in turn can cause increased blood viscosity and serum bilirubin, respectively. Given that bilirubin can be antioxidant or prooxidant, it would therefore be a factor of predominance. That is, (a) pro-oxidant bilirubinaemia and blood viscosity could be associated and positively correlated; or (b) reducing WBV due to haemolysis- induced anaemia and increasing antioxidant bilirubin could cause negative correlation. Second, oxidative stress may be assessed using Lp(a), but whether the latter is associated with blood viscosity is not yet established. Thus, we hypothesize that an increase in plasma Lp(a) level as an indirect index of oxidative stress is associated and positively correlates with WBV.

Objective

This study investigated whether decreased haemoglobin level or an increase in WBV is associated with increase in bilirubinaemia and/or plasma Lp(a) concentration in a general population of patients. Of particular interest was whether bilirubinaemia and/or Lp(a) level should be investigated for use as indirect indices of oxidative stress in clinical practice.

Assumption

There is a problem regarding variation in reference values for Lp(a), especially due to racial differences. It was assumed that this variation in reference values in different populations does not impact upon the study. The use of hyperbilirubinaemia and/or Lp(a) as indirect indices of oxidative stress is yet to be established.

Material and methods

This work is part of a clinical laboratory-based Biomedical Science Research supported materially by Albury South West Pathology - a unit of the Western Pathology Cluster of NSW Health, Australia. The Ethics Committee of the Area Health Service approved the use of the de-identified data

![Hypothetical association between bilirubinaemia, haemoglobin, Lp(a), and blood viscosity -- callouts indicating known points of association in the pathophysiology.](image-url)
The database comprised archived clinical pathology data from January 1999 to December 2008. All tests were performed at the Albury laboratory of South West Pathology, except for Lp(a) which were sent to Newcastle Hunter Area Pathology Service, Newcastle. Bilirubin measurement in this study refers to total conjugated and unconjugated bilirubin. Lp(a) test results in the 10-year period were obtained from the laboratory information system and audited. Sixty-eight cases, comprising only adults, that had data for full blood counts (FBCs) and liver function test (LFT) parameters were selected.

HCT and TP were used to determine WBV at high shear rate by extrapolation method, as follows:

\[
\text{High shear rate: } \text{WBV}(208 \text{ seconds}^{-1}) = (0.12 \times \text{HCT}) + 0.17(\text{TP} - 2.07)
\]

where HCT = haematocrit (%) and TP = serum total proteins (g/l).

The data (n = 68) were first ranked and categorized into quartiles based on haemoglobin levels, and recategorized based on WBV. In order to evaluate possible association with serum Lp(a) and bilirubin, multivariate (MANOVA) and univariate (ANOVA) analyses were performed to determine difference between subgroups using S-Plus.

Correlation analysis was performed using the CORREL function in Microsoft Excel. Parameters that make up the LFTs and haematological indices were included. The intention was to ascertain whether any of the parameters would be correlated and/or statistically significantly related. In order to visualize possible changes in haemoglobin level and WBV associated with serum bilirubin and Lp(a) levels observable in any follow-up of individuals, three cases that had two sets of results each, were reviewed.

Results

The description of the data is presented in Table 1. Among the four parameters of interest, distributions are accepted to be normal (kurtosis < 3), but Lp(a) concentration is very widely distributed (Table 1). Therefore, Lp(a) values were transformed into the log inverse for analysis.

In the evaluation of changes in various parameters with incremental changes in (ranked) haemoglobin levels, MANOVA presented statistical significance between subpopulations in 1st vs. 4th quartile (P = 0). Univariate analysis (ANOVA fixed factor) of Lp(a) and serum bilirubin (SB) presented statistically significant difference in the former, but not in the latter (Fig. 2).

In the evaluation of changes in various parameters with incremental changes in (ranked) WBV, MANOVA demonstrated statistical significance (P = 0). Univariate analysis (ANOVA fixed factor) gave a statistically significant not in Lp(a) (Fig. 3).

The outcome of indicate positive correlation bilirubinaemia, but showed negative correlation with Lp(a), but not with bilirubinaemia (Table 2).
Case evaluations

The three cases reviewed indicates that when Lp(a) levels were higher, all three patients had lower levels of serum bilirubin, while two-thirds had higher WBV and lower haemoglobin (Table-3).

Discussion

It is known that oxidative stress is associated with increase in hyperviscosity, but not necessarily with low HCT/haemoglobin. We have investigated how serum Lp(a) level and bilirubinaemia, as potential indirect indicators of oxidative stress, would be associated and/or correlated with WBV and haemoglobin. Our results show that hyperbilirubinaemia, but not Lp(a), is associated and positively correlated with WBV. It also shows albeit conversely that Lp(a) is associated and negatively correlated with haemoglobin concentration.

It suggests that as oxidative stress is more associated with increasing WBV and not decreasing HCT/haemoglobin, so also is bilirubinaemia. Further, the results show that among all components of the LFT panel, bilirubinaemia is most correlated and statistically significantly related to WBV. There is a report indicating that patients with cholestatic jaundice have increased WBV. Therefore, the observation reported here affirms that hyperbilirubinaemia is positively correlated to WBV. Bearing in mind the antioxidant potential of bilirubin, the positive correlation with WBV could also suggest a possibility of pro-oxidant bilirubin overwhelming the antioxidant property.

Another interesting aspect of the study is whether Lp(a), as an indirect index of oxidative stress, is more associated with either anaemia or WBV. The pertinent observation is a statistically significant inverse relationship between haemoglobin and Lp(a). It could be inferred that the statistically significant negative relationship is due to oxidative stress concomitantly inducing membrane lipoprotein metabolism leading to increased Lp(a); and haemolysis causing a reduction in haemoglobin level (Fig. 1). However, we note that the hypothesis of correlation between Lp(a) and WBV failed.

Further, a critical visual review shows that there is an increase in bilirubin level associated with increase in haemoglobin concentration (Fig. 2), but this was not significant. This observation is in line with the report that erythroid apoptosis is not correlated with bilirubinaemia. In two of the three cases, which were reviewed to check the assumption that the racial differences in normal level of Lp(a) may not impact on changes to be observed in any follow-up of individuals, it is observed that WBVs were higher and haemoglobin concentrations lower when the serum Lp(a) concentration increased. Thus, the anecdotal cases indicate that increase in Lp(a) may be associated with increase in WBV, but correlation analysis in the general population did not corroborate.

Correlation does not always imply causation and some studies have shown that haemolysis may not contribute significantly to other pathophysiology associated with oxidative stress. Conversely, non-significant correlation does not always imply lack of relationship. In this study, Lp(a) levels showed negative correlation with haemoglobin, but not significantly related to bilirubin or WBV. However, the propensity of oxidative stress to cause haemolysis- induced hyperbilirubinaemia and hyperviscosity is demonstrated by the positive association as well as correlation between bilirubin and WBV. Further, the statistically significant inverse correlation between haemoglobin and Lp(a) in the study tends to lend weight. Therefore, each of the four variables has demonstrated an explainable correlation with at least one of the other three.

Study limitations

There are several limitations in this study. First, the participants were de-identified. As the outcome of this study provides them no direct or immediate benefit, contact with patients or their clinicians was not made. Information on disease condition, clinical management, drugs, and presence of metabolic disorders, malignancy, or coagulation disorders were not accessed. Second, serum bilirubin measured in this study was the total fraction with no differentiation between unconjugated (USB) or conjugated bilirubin. The data do not include neonates who are typically not tested for Lp(a) and we acknowledge this as a limitation especially, due to issues such as (i) USB crossing the blood-brain barrier and affecting the integrity of microvascular endothelial cell monolayers through oxidative stress, and (ii) hyperbilirubinaemia vs. anaemia with regard to causation. High bilirubin from haemolytic, pre-hepatic processes has a high unconjugated content and bilirubin from post-hepatic or disturbed bilirubin transport processes would be mostly conjugated. This becomes confounded when there is haemolysis from anaemia with concomitant liver disease. This study has other limitations in that there were no obvious cases of anaemia or hyperviscosity in the study population. Further, a small fraction of females were tested and their haemoglobin levels are generally lower in comparison with that of males.

Conclusion

This study has determined that bilirubinaemia and blood viscosity are associated and positively correlated, while haemoglobin and Lp(a) are associated and negatively correlated. The implication is that the pro-oxidant property
of serum bilirubin (as an indirect index of oxidative stress) can be used in combination with WBV (as an index of concomitant macrovascular effect of oxidative stress) to assess oxidative damage. Further investigation to corroborate this report is recommended.

References


Appendix E: Algorithm for whole blood viscosity: Implication for antiplatelet bleeding risk assessment

Algorithm for whole blood viscosity: implication for antiplatelet bleeding risk assessment

Ezekiel U. Nwose & Eugene G. Butkowski

School of Psychological & Clinical Sciences, Charles Darwin University, Northern Territory
South West Pathology Service, NSW Health, New South Wales

Abstract

A series of evaluations on whole blood viscosity (WBV) issues tried to elucidate the sensitivity, specificity and usefulness of the laboratory parameter in clinical practice. The aim of this article is to postulate (1) how to use routine clinical laboratory tests to derive WBV, and (2) the usefulness of WBV in evidence-based practice for determination of the therapeutic risk of bleeding. The study used 10 years of archived clinical pathology data from South West Pathology. WBV was derived from calculations incorporating haematocrit and total protein and extrapolation chart and reference values were developed. Association of and/or changes in WBV level with acetylsalicylates, C-reactive protein (CRP), D-dimer, Erythrocyte Sedimentation Rate (ESR), Faecal Occult Blood (FOB), homocysteine, International Normalised Ratio (INR), leucocytosis, leukapheresis, and platelet levels were determined. Whether there are differences between grades of WBV levels in the laboratory indices of diabetes, dyslipidaemia and renal function test were also investigated. A comparison with diagnostic digital method was also performed. One possible false assumption is that WBV (akin to CRP and ESR) is too sensitive and not a specific marker for the diagnosis of a pathological condition. Our findings may refute this notion. Interestingly, lower WBV levels are significantly associated with higher INR and acetylsalicylate levels. The observations from this study also elucidate the potential of WBV for evidence-based pathology to support a decision of antiplatelet medication, akin to INR in anticoagulant therapy. The clinical laboratory method postulated here can be performed in any facility that performs routine biochemistry and haematology.

Keywords: antiplatelet therapy, bleeding risk, evidence-based pathology, whole blood viscosity
Introduction

Virchow’s triad has been an established concept of three broad factors that ultimately lead to, and/or result from thrombosis vis-a-vis cardiovascular complications (Bagot and Arya, 2008, Lowe, 2003). Each factor represents a subclinical vascular process, which in turn is indicated by a clinical pathology index (Table 1).

Table 1: Virchow’s triad, corresponding vascular process and pathology index

<table>
<thead>
<tr>
<th>Virchow’s triad</th>
<th>Vascular process</th>
<th>Pathology index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atherothrombosis</td>
<td>Blood coagulability/fibrinolysis</td>
<td>Plasma D-dimer</td>
</tr>
<tr>
<td>Endothelial dysfunction</td>
<td>Blood vessel injury or irritation</td>
<td>Homocysteine</td>
</tr>
<tr>
<td>Stasis</td>
<td>Blood flow changes</td>
<td>Blood viscosity</td>
</tr>
</tbody>
</table>

Antiplatelet therapy such as aspirin is known for its cardio-protective effects as well as its potential risk to cause bleeding complications. Hence, there are recommended guidelines for usage (Colwell and American Diabetes Association 2004, Bertrand 2008, U.S. Preventive Services Task Force 2002). One of the major confounding factors to the risk of bleeding is whole blood viscosity (WBV) (Mannini et al 2006, Cecchi et al 2009). Colloquially, antiplatelet agents are blood thinners (Weinberg 2011). Blood with a less than normal viscosity would bleed/flow faster compared to high viscosity blood (Berman et al 1994, Amir and Krauss 1973). Therefore, a logical contraindication for antiplatelet therapy, to avoid associated bleeding risk would be indicated by the WBV status.

The therapeutic assessment of the ‘risk of bleeding’ is possibly non-existent (Rodak et al 2012, Sabitha et al 2008). There have been various platelet function test (PFT) methods including bleeding time and platelet aggregometry. It has been acknowledged that clinical laboratories over the years have been performing PFT by different aggregometry methods with little or no standardization (Breddin, 2005). Hence, the Clinical and Laboratory Standards Institute recently developed a guideline to address this void (Christie et al 2009). However, it is pertinent to appreciate
that the guideline is about standardizing aggregometry methods per se. The guideline does not recommend a particular method for diagnosis of platelet disorders or choice of a therapeutic agent (Christie et al 2009). A critical review of the of the principles and utility of platelet aggregometry will show that it is limited as a therapeutic drug monitoring tool for investigating whether a patient is resistant, responsive or nonresponsive to therapy (Favaloro 2008, von Beckerath et al 2010). Patients would have taken the medication prior to testing for resistance or responsiveness. This is quite different from testing prior to medication whether a patient would be at risk of bleeding. Therefore, there is still a void in PFT with reference to assessing bleeding risk or contraindication of antiplatelet therapy. This technical note addresses a practical issue of evidence-based compliance to guidelines on the use of antiplatelet therapy in clinical practice. The objectives are to present (1) an algorithmic method for WBV testing; and (2) the value of the pathology test when appropriated.

Methods

This evaluation of de-identified data was approved by the HREC of South West Pathology Albury. The de-identified data pool for 1999—2008 were acquired as part of laboratory-based research agenda from the Auslab Laboratory Information System (Nwose et al 2010). Using an arithmetic formula: ‘WBV (208 Sec-1) = 0.12 x HCT + 0.17(TP—2.07)’ (Tamariz et al 2008), WBV at high shear stress was determined from various possible pairs of haematocrit and total proteins. Extrapolation chart and reference values were developed (Fig. 1), based on the formula, with HCT and TP being haematocrit (%) and serum total proteins (g/L) respectively (Nwose 2010). The results obtained from the extrapolation method were compared with a digital method (Nwose and Richards 2011).

To evaluate the specificity and sensitivity of WBV to associated pathophysiology, the associations of WBV with several laboratory parameters were performed. Correlation with CRP and ESR was evaluated to determine whether WBV would be equally sensitive to similar clinical conditions. Changes in the level of WBV were compared with those patients on acetylsalicylate therapy and results of FOB to determine correlation and specificity for ‘risk of bleeding’ to aspirin. Also, changes in the level of WBV were compared with those of INR to determine specificity for ‘risk of bleeding’ associated with anticoagulants and antplatelets.

Results

The result of the investigation of how WBV correlates with routine acute phase inflammation parameters showed that hyperviscosity was observed in 2.9% of increased CRP cases, and 2.7% of raised ESR results. The results show that hyperviscosity is not frequently observable in acute phase inflammation. The results also show that on average, normoviscosity is concordant with 85.3% of normal CRP and 93.8% of normal ESR reports. Further, the results indicate that there is more hyperviscosity, but less hypoviscosity, in elevated than in normal CRP and ESR subpopulations (Table 2).

On investigating whether WBV is associated with the INR and platelet count, it was observed that WBV is directly and inversely associated with platelet counts and INR respectively (p<0.002) (Fig. 2).

Since aspirin measured as acetyl-salicylate is used in the management of stasis, of which blood viscosity is an index, it was hypothesized and investigated whether WBV would be inversely related to the
blood level of acetylsalicylate. The hypothesis was proven as it was observed that lower WBV is statistically significantly associated with higher acetylsalicylate level (p < 0.002; Table 3) (Nwose and Cann 2010).

When investigating the prevalence of hypoviscosity in diabetes, dyslipidaemia and renal failure with a view to determine the proportion of chronic disease patients who may not require antiplatelet therapy, it was observed that up to 97.5% of cases investigated for chronic diseases had a normal to high level of WBV, which could indicate patients may benefit from antiplatelet therapy (Fig. 3).

![Figure 3. Comparison of creatinine, glycemia and lipidaemia between WBV groups *averaged standard deviation for TC/HDL: 1.5, FBS: fasting blood sugar (averaged Standard deviations: 1.8), HDL: high density lipoprotein, S. Cr: serum creatinine (averaged standard deviations: 4.7, TC: total cholesterol, WBV: whole blood viscosity]*

When investigating the prevalence of hypoviscosity in diabetes, dyslipidaemia and renal failure with a view to determine

| Table 2: Prevalence of hypoviscosity in abnormal and normal sub-populations of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) |
|---------------------------------|-----------------|-----------------|

<table>
<thead>
<tr>
<th>CRP n</th>
<th>1270</th>
<th>3.6</th>
<th>3.9</th>
<th>92.5</th>
<th>4355</th>
<th>23.7</th>
<th>3.4</th>
<th>72.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>2607</td>
<td>4.2</td>
<td>3.8</td>
<td>92.0</td>
<td>833</td>
<td>20.4</td>
<td>2.3</td>
<td>77.3</td>
</tr>
<tr>
<td>2008</td>
<td>3472</td>
<td>4.7</td>
<td>2.8</td>
<td>92.5</td>
<td>530</td>
<td>18.1</td>
<td>2.3</td>
<td>76.6</td>
</tr>
<tr>
<td>2009</td>
<td>1226</td>
<td>6.7</td>
<td>3.1</td>
<td>90.2</td>
<td>365</td>
<td>20.9</td>
<td>3.5</td>
<td>70.3</td>
</tr>
<tr>
<td>1990</td>
<td>124</td>
<td>23.2</td>
<td>2.5</td>
<td>59.3</td>
<td>399</td>
<td>17.9</td>
<td>3.0</td>
<td>79.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CRP n</th>
<th>6710</th>
<th>2.0</th>
<th>4.7</th>
<th>93.3</th>
<th>4478</th>
<th>7.9</th>
<th>3.6</th>
<th>85.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>4555</td>
<td>1.8</td>
<td>3.2</td>
<td>95.0</td>
<td>3584</td>
<td>7.2</td>
<td>2.6</td>
<td>90.2</td>
</tr>
<tr>
<td>2008</td>
<td>4378</td>
<td>2.8</td>
<td>2.7</td>
<td>94.3</td>
<td>3264</td>
<td>10.3</td>
<td>2.2</td>
<td>87.3</td>
</tr>
<tr>
<td>2009</td>
<td>4794</td>
<td>4.1</td>
<td>2.5</td>
<td>93.4</td>
<td>2866</td>
<td>13.2</td>
<td>1.9</td>
<td>84.9</td>
</tr>
<tr>
<td>1990</td>
<td>1582</td>
<td>4.6</td>
<td>2.5</td>
<td>92.9</td>
<td>1135</td>
<td>15.1</td>
<td>3.0</td>
<td>81.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Body Mass Index</th>
<th>BMI</th>
<th>27.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>27.2</td>
<td>16.11</td>
<td>18.19</td>
</tr>
<tr>
<td>27.4</td>
<td>16.51</td>
<td>17.16</td>
</tr>
</tbody>
</table>

| Table 3: WBV vs. salicylate statistics |

<table>
<thead>
<tr>
<th>Area</th>
<th>Salicylate</th>
<th>WBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>5.22</td>
<td>16.61</td>
</tr>
<tr>
<td>Median</td>
<td>4.4</td>
<td>16.69</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>3.97</td>
<td>1.72</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.93</td>
<td>8.38</td>
</tr>
<tr>
<td>Maximum</td>
<td>4.28</td>
<td>21.17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analyses</th>
<th>Mean WBV Levels</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top vs. Bottom</td>
<td>Higher Sal* vs. Lower Sal*</td>
<td>0.0001</td>
</tr>
<tr>
<td>5th vs. 1st</td>
<td>16.51</td>
<td>17.16</td>
</tr>
<tr>
<td>Top vs. Bottom halves</td>
<td>4.40</td>
<td>16.82</td>
</tr>
</tbody>
</table>

Key: *Salicylate, Higher salicylate level = lower WBV and vice versa
Discussion

The results from scientific research can quickly and erroneously be categorized as relevant or irrelevant. WBV falls into the latter category and attempts to discuss WBV most often turns out a waste of time (British Medical Journal blog: http://blogs.bmj.com/bmj/2009/04/24/ richard-smith-on-countering-the-%E2%80%9Cwicked- problem%E2%80%9D-of-the-chronic-disease-pandemic/). Another reason for discussion or research on WBV not being given credence is the notion that WBV is too sensitive or non-specific. This is especially so considering the speculation of possible minor changes in blood viscosity without known physiologic consequence (Smith and La Celle 1982). However, the findings from this study show three distinct points as follows:

WBV is not too sensitive: WBV may not necessarily be too sensitive to related disease conditions. Acute inflammation can lead to stasis by normal physiological response. The cumulative prevalence of the different categories of WBV observed among sub-populations of normal and raised CRP and ESRs indicates that while normal WBV is most prevalent in the general population, hyperviscosity is not prevalent in acute phase inflammation (Table 2). The observations indicate that WBV is not arbitrarily as sensitive as CRP or ESR.

WBV is specific, not non-specific: The results indicate that salicylate medication (aspirin) reduces WBV level. Lower or no salicylate would mean relatively higher WBV level (Table 3). This observation proves the hypothesis that antiplatelet prophylaxis or therapy is meant to reduce WBV. While this may have been implied or known (Higgins 2006), the issue is that INR is mainly used to measure certain haemostatic factors and to monitor anticoagulant therapy but is not assessed for chronic disease patients who merely require antiplatelet prophylaxis. Neither is WBV assessed on the chronic patients who are being managed with antiplatelet prophylaxis or therapy, nor is the risk of bleeding complication of less concern (Sabitha et al 2008).

WBV indicates antiplatelet prophylaxis is safe for most chronic disease patients: Another very positive finding is evidence-based pathology that most patients with chronic diseases would not be diagnosed with hyperviscosity or hypoviscosity i.e. the majority have normoviscosity (Fig. 3). It would be reasonable to presume that most patients attending pathology for routine creatinine, glucose, lipid profile monitoring could be on medication including antiplatelet prophylaxis or therapy. A study had reported that the prevalence of antiplatelet use was 54% overall, while another indicated 84% usage among a diabetic cohort (Miller et al 2005, Bulatova et al 2007), which would lower WBV in this population. Therefore, observations of normal to high blood viscosity in 97.5% of the patients is an indication of those who are benefiting from therapy without WBV falling to a level that predisposes to risk of bleeding.

Implications for clinical practice

Discussion in relation to practice: There is doubt over the evidence base for aspirin in chronic disease management (Walsh and Spurling 2008), which would benefit from the discussion on what Australia can do to increase Evidence based Pathology (The Royal College of Pathology 2010). It is known that WBV attenuates the efficacy of antiplatelet agents (Mannini et al 2006). That is, WBV is supposed to be duly assessed and monitored for those who going to be, or are being treated with this therapy (akin to INR monitoring of warfarin). Perhaps the limitation has been that test methodology is not readily available. It is
therefore imperative to promote the availability of a algorithmic method to easily determine WBV.

Antiplatelet agents are recommended with emphasis that individuals with cardio-embolic or athero-thrombotic condition, stroke or TIA can receive an antiplatelet agent to reduce the risk of recurrent stroke on the condition of patient’s tolerance and no contraindication (Cucchiara and Messe 2011) and there are guidelines published from the American Heart Association/American Stroke Association (AHA/ASA) and the American College of Chest Physicians (Albers et al 2008, Furie et al 2011).

While there are platelet function tests (PFT) options to measure patient’s tolerance, the consideration for “no contraindication” has yet to be established. The original ‘bleeding time’ method is almost obsolete in modern day laboratory. Platelet aggregometry is becoming more common, but not without its limitations (Rodak et al 2012). One of the pre-analytical difficulties, and probably the most important in the quality assurance and control, is the accessibility, availability and transport of a fresh blood specimen containing functional platelets (Favaloro 2009).

There are issues relating to bleeding complications, non-response, resistance and treatment failure of antiplatelet therapy such as aspirin or clopidogrel (Hennekens et al 2011). Perhaps, it is pertinent to separate the first (bleeding complications) from the other three especially in terms of (i) medication prior to testing for resistance, responsiveness or treatment failure; versus (ii) testing prior to medication whether a patient would be at risk of bleeding. In the spirit of “what can Australasia do to increase evidence-based pathology” (The Royal College of Pathology 2010), this paper calls for consideration to integrate existing evidence for the underutilised test, WBV.

Implications for laboratory managers: Beside the benefit of WBV assessment to the patients who may be at risk of bleeding, a valuation of revenue accruable from such assessment indicated that the laboratory service would have generated AU$615,272.00 for a regional lab in a 3-year period, including AU$39,304.00 from the diabetes clients only (Nwose et al 2009). A brief cost analysis shows that revenue from a WBV test alone is far more than it costs to generate the result by the algorithmic method. This is beside the advantage of providing a service that has eluded remote communities.

Conclusion

This paper puts forward an extrapolation method for WBV test. The notion that WBV is too sensitive or non-specific marker is refuted and the issue of evidence base pathology for antiplatelet therapy is addressed. The concern for bleeding risk of antiplatelet therapy is also differentiated from the problems of responsiveness or resistance to therapy.

Acknowledgement

This study was done with material and support of the management of the South West Pathology Service, Albury.

References


New Guidelines for Diagnosis of Gestational Diabetes: Pathology-Based Impact Assessment

Ezekiel Uba Nwose¹,³, Ross Stuart Richards², Phillip Taderera Bwititi², Eugene George Butkowski¹

¹Charles Darwin University, NT Australia, ²Charles Sturt University, NSW Australia, ³NSW Health Pathology, NSW Australia

Abstract

Background: A recent study indicated an average of 19.5% abnormal oral glucose tolerance in antenatal clients per year. Aim: The purpose of this study was to determine the impact on gestational diabetes cases due to new guidelines for diagnosis and classification of hyperglycaemia in pregnancy. Materials and Methods: This study reviewed the archived clinical pathology data on oral glucose tolerance tests performed between January 1999 and December 2008 on antenatal clients (N = 615). The cases were reviewed to determine changes if any in percentage of gestational diabetes due to new guidelines. Results: Over the 10 years period, a yearly average of additional 10.8% antenatal cases suggestive of gestational diabetes was observed due to the new recommended thresholds. Further, the average yearly incidence would have increased from 8.8 cases to 16.2 cases, which translates to almost 46% increase in the prospective numbers of gestational diabetes. Conclusions: This report presents the extent of how the new recommended guidelines for diagnosis and classification of hyperglycaemia in pregnancy could increase the prevalence of gestational diabetes mellitus and allows for planning the costs that would be attendant to the full implementation of the new guidelines.

Keywords: Gestational diabetes, Impact evaluation, New guidelines, Oral glucose tolerance test, Pathology-based evidence

Address for correspondence: Dr. Ezekiel Uba Nwose, School of Psychological and Clinical Sciences, Casuarina Campus, Yellow 2.2.58, Charles Darwin University, NT 0909, Australia. E-mail: uba.nwose@cdu.edu.au
Introduction
A report from Norway published in 1994 in the Scandinavian Journal of Primary Health Care indicated a prevalence of up to 45% abnormal oral glucose tolerance test (OGTT). Although OGTT is highly criticized as inconsistent, inconvenient and poorly reproducible, it still adds predictive advantage to the use of fasting blood glucose level alone. Thus, it is still the gold standard, especially to make gestational diabetes diagnoses. However, 18 years since the Norwegians’ report, corroborative data from other countries are lacking, including pathology-based evidence regarding the actual prevalence of gestational diabetes.

In 2010, the International Association of Diabetes and Pregnancy Study Groups issued new guidelines for diagnosis and classification of hyperglycaemia in pregnancy. That is, diagnosis of gestation diabetes mellitus (GDM), which is defined in this report as any degree of glucose intolerance with onset or first recognition during pregnancy. A concern is that there is a subgroup of overt diabetics in antenatal patients who are at risk of adverse pregnancy outcomes, but there seems to be no clear correlation to hyperglycaemia. Thus, the new guidelines introduced a brand set of thresholds and recommendations. Since the recommendations, there have been series of comments, especially on the potential increase in number of GDM, the same antenatal subpopulation (N=615) was further reviewed. Based on the recommended threshold, data were sorted as follows:

1. First, by the diagnostic report sent to clinicians: for each year, those reported as suggestive of GDM were ranked on top. The counts were evaluated as percentage of antenatal subpopulations to determine the prevalence of GDM diagnosis (% Dx).
2. Second, by the new threshold: those not reported as suggestive of GDM, but had fasting blood glucose level of 5.1 mmol/L (92 mg/dL), 1 h postprandial glucose level of 10.0 mmol/L (180 mg/dL) and/or a 2-h postprandial glucose level of 8.5 mmol/L (153 mg/dL) were identified. The counts were evaluated as percentage of antenatal subpopulations to determine the prevalence of "DM diagnosis based on new guideline" (% Pro).
3. The absolute numbers that made up "% Dx" and "% Pro" were then pooled together to determine the might-have-been prevalence or prospective GDM diagnosis (% Pro) by the new guidelines. Lastly, the fraction of the "other" in the "Pro" for the 10 years period is expressed as the potential impact of the new guidelines.

Materials and Methods

Study setting
This work was done at the South West Pathology Service of New South Wales (NSW) Health Australia, as part of a Translational Biomedical Science Research initiative. The pathology operates a Laboratory Information System (LIS) that could only be access by permission and with a password. The Ethics Committee of the Area Health Service approved the acquisition and use of de-identified database from the LIS. The database comprises 10 years of archived clinical pathology data from January 1999 to December 2008. All OGTT (glucose load 75 g) performed in the 10 years period were audited. This included 5126 cases, of which 615 were antenatal. The OGTT results from the 10 years under review, including all those selected as GDM, had blood glucose levels for the three points (fasting, as well as 1 h and 2 h postprandial).

Source of information and definition on gestational diabetes
As this study was based on de-identified data, and no immediate benefit for the study clients was envisaged, no personal contact was made or letter from clinicians solicited (N=615). OGTT were performed on antenatal patients. There was no record indicating previous diabetes or pre-diabetes on these clients. The source of information about GDM for this evaluation was from the de-identified OGTT reports in the LIS. The definition of GDM was based on the clinical pathology reports sent out to the clinicians, i.e. cases that were reported as indicating gestational diabetes were evaluated as percentage of the 615 antenatal subpopulations.

Analyses
To assess the impact of the new guidelines on potential increase in number of GDM, the same antenatal subpopulation (N=615) was further reviewed. Based on the recommended threshold, data

Table 1: Summary statistics of all oral glucose tolerance test reports for antenatal subpopulation

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>9</td>
<td>42</td>
<td>33</td>
<td>32</td>
<td>14</td>
<td>13</td>
<td>15</td>
<td>24</td>
<td>129</td>
<td>304</td>
</tr>
<tr>
<td>GDM</td>
<td>2</td>
<td>11</td>
<td>8</td>
<td>10</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>12</td>
<td>34</td>
</tr>
<tr>
<td>Normal</td>
<td>7</td>
<td>31</td>
<td>21</td>
<td>20</td>
<td>13</td>
<td>9</td>
<td>10</td>
<td>21</td>
<td>114</td>
<td>269</td>
</tr>
<tr>
<td>TNC</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>% Pos</td>
<td>22.2</td>
<td>26.2</td>
<td>24.2</td>
<td>31.3</td>
<td>7.1</td>
<td>30.8</td>
<td>20.9</td>
<td>12.5</td>
<td>9.3</td>
<td>11.2</td>
</tr>
</tbody>
</table>

GDM: Gestation diabetes mellitus; TNC: Test not completed; N: Total number; Pos: Abnormal

The summary statistics of the results is presented in Table 1. The yearly average of 19.5% was reported as gestational diabetes [Table 1].
evaluations is the "normal" reports; how many of them would have been reported as GDM based on the new recommended guideline or threshold. Using the new guidelines, the results show an additional yearly average of 10.8%, and the 10 years additive of 12% (74 out of 615) would have been reported as GDM. That is, the incidence or number of GDM cases would have increased from 88 to 162 [Table 2], which translates to approximately 46% impact [Figure 1].

Discussion

The main focus of this study was to investigate and quantify the potential impact of the new guideline thresholds for diagnosis or classification of hyperglycaemia in pregnancy. Initial review shows that by the pathology's protocol, yearly average of 19.5% of antenatal OGTT tests were reported as gestational diabetes.[13] A further review of the case reports that were classified as normal [Table 1], but this time using the new recommended thresholds, revealed that an additional yearly average of 10.8% would have been reported as gestational diabetes [Table 2].

A cursory look at the descriptive statistics or the information on Table 2 may not reveal the full impact of the new recommendations on prospective incidence of GDM. For instance, it could be hastily translated to be *10.8/(19.5 + 10.8) x 100 = 35.6%.* Another potential hasty translation could be to take only 1 or 2 years, but the results show unevenness in the yearly impact. A critical evaluation shows that the increase in number from 88 to 162 translates to 45.7% [Figure 1].

It is pertinent to note one of the reviews of the new criteria that the total incidence of gestational diabetes in the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) population was 17.8%,[7] which is less than half of what is being reported here. This is probably a reflection of the different populations studied. Nevertheless, the significance comes to bear in estimating and planning for the management of GDM in the population, and reinforces the call to study local populations because of geographical and social impacts among others.

Conclusion

We report a level of prevalence of abnormal OGTT that has yet to be fully appreciated. We also report pathology-based evidence that the new guidelines for diagnosis and classification of hyperglycaemia could increase the GDM cases by as much as 46%. The importance of this report is in the epidemiological information necessary for estimating the cost of adopting criteria.

Acknowledgments

The acquisition of archived clinical pathology data used in this work was permitted and supported by the management of South West Pathology Service of NSW Health Australia. There is no financial interest.

References

9. Hadar E, Hod M. Establishing consensus criteria for the


Appendix G: Hyperglycaemia, oxidative stress and inflammatory markers

DOI: 10.1080/ 13510002.2016.1215643
Hyperglycaemia, oxidative stress and inflammatory markers

Butkowski, Eugene G.\textsuperscript{a}, and Jelinek, Herbert F.\textsuperscript{a,b,c}

\textsuperscript{a} School of Community Health, Charles Sturt University, Albury, Australia.
\textsuperscript{b} School of Medicine, University of New South Wales \textsuperscript{c} Faculty of Medicine and Health Sciences, Macquarie University, Sydney, Australia

Address for Correspondence
Herbert F. Jelinek
School of Community Health
Charles Sturt University
Albury 2640
Australia
E: hjelinek@csu.edu.au

Abstract

\textbf{Introduction:} The increasing prevalence of hyperglycaemia implicates a state of oxidative stress and inflammation. Traditional and emerging biomarkers associated with increasing hyperglycaemia were assessed to clarify the role they play in hyperglycaemia.

\textbf{Results:} 309 participants attending a rural diabetic screening program were categorised into control and quintile groups based upon glucose levels: 1\textsuperscript{st} quintile - <4.5mmol/L and 4\textsuperscript{th}, 5\textsuperscript{th} quintile - >6.1mmol/L. Significant results were obtained for anthropometric data and biochemical markers - glucose, HbA1c and total cholesterol ($p < 0.001$); oxidative stress: glutathione ($p < 0.001$), glutathione/glutathione disulfide and 8-hydroxy-2-deoxyguanosine ($p < 0.05$). Interleukin -1\textbeta and inflammatory marker ratios IL-6/IL-10, IL-1\textbeta/IL-10, MCP-1/IL-10, IGF-1/IL-10 and IL-6/IL-1\textbeta were significant ($p < 0.05$).

\textbf{Conclusion:} This study provided further evidence that inflammatory and oxidative stress biomarkers may contribute to diagnostic information associated with preclinical increases in BGL. Further we have provided a unique study in the analysis of ratios of inflammatory biomarkers and correlations with increasing BGL.
Keywords: Type 2 Diabetes Mellitus, impaired fasting glucose, prediabetes, cardiovascular disease, oxidative stress, risk factors, body mass index, glutathione, glutathione disulfide, 8-hydroxy-2’-deoxyguanosine, interleukin 1β, interleukin-6, interleukin 10, monocyte chemoattractant protein 1, insulin like growth factor 1

Introduction:

Alterations to homeostatic disturbances in glucose metabolism and resultant hyperglycaemia are causative factors for Type 2 diabetes mellitus (T2DM). Chronic sustained hyperglycaemia also results in micro and macrovascular complications occurring through a number of mechanisms of which oxidative stress and inflammatory changes via the innate immune system have increased in interest for medical diagnostics (1, 2). Impaired fasting glucose (IFG) may lead to the development of T2DM and cardiovascular disease (CVD), associated with increased oxidative stress (3) along with the ensuing chronic subclinical inflammatory processes apparent with developing insulin resistance (4). The challenge is to prevent complications associated with IFG of which approximately 30% will remain undiagnosed for a significant period of time (5). The International Diabetes Federation have recognised the importance of the traditional biomarkers (general biochemistry): blood glucose (BGL), haemoglobin A1c (HbA1c) and lipid studies along with life style improvement and drug treatment regimens to combat T2DM and CVD. There is also a recognition that lower blood glucose levels (<7.8mmol/L) have lower rates of all major event point diabetic complications (6). However pathophysiological processes already occur with minor increases in BGL. Traditional biomarkers as predictors for T2DM and CVD remain suboptimal in indicating disease progression especially when BGL is in the prediabetic range. This study further investigated the use of emerging oxidative stress and inflammatory markers in conjunction with traditional biomarkers and anthropometric data to establish if there was any association with blood glucose levels of <4.5mmol/L (1st quintile) and >6.1mmol/L (4th, 5th quintile) and their utility as potential predictors of T2DM and CVD development. Patients with a BGL indicative of the prediabetic state (>6.1 mmol/L) demonstrate an increased propensity to develop T2DM and CVD and emerging biomarkers are of clinical interest in improving risk of diabetes progression. Our hypothesis was that there is a significant difference between inflammatory markers and oxidative stress markers at raised blood glucose levels to control BGL
levels and indicate the usefulness of these markers as a diagnostic aid in T2DM and CVD prevention.

Chronic hyperglycaemia is considered to be a major causative factor in the establishment of microvascular and macrovascular complications observed in T2DM. Reactive oxygen species (ROS) as a result of hyperglycaemia, are known to damage nucleic acids, lipids and proteins with the degree or extent of damage related to the duration of hyperglycaemia (7, 8). The associated pathophysiological mechanisms which occur are suggestive of the excess generation of oxygen and nitrogen species and concomitant oxidative stress (9). Oxidative stress (OS) is initiated by hyperglycaemia as a result of increased circulating intracellular and extracellular free radical levels (10, 11). Glutathione (GSH), is a responsive ubiquitous antioxidant and detoxifier of excessive free radicals and reactive oxygen species (10, 12) and readily oxidised to glutathione disulfide (GSSG) making it the major physiological redox reaction (13). Any oxidation of GSH and a consequential increase in GSSG leads to changes in the GSH:GSSG ratio, which can be utilised as a useful marker in determining an antioxidant status (14). As decreased levels of GSH have been observed as a consequence of hyperglycaemia, primarily as a result of cysteine depletion and loss of cysteine assisted membrane transport mechanisms across the red blood cells, cellular oxidative stress may increase (15, 16). Oxidative damage to DNA and RNA occurs due to nuclear free radical formation as a result of hydrogen peroxide oxidation. The interaction of the hydroxyl radical with the DNA nucleobase guanine forms the product 8-hydroxyguanine (8-OHGua) or the nucleoside 8-hydroxy-2'-deoxyguanosine (8OHdG) (17). Increased levels of 8OHdG have previously been shown to be useful biomarkers in the assessment of atherosclerosis and T2DM (18). Oxidative stress and inflammation go hand in hand as a result of hyperglycaemia and increased reactive oxygen species (ROS) (19, 20). There is therefore an imperative to observe levels of both oxidative and inflammatory markers in the investigation of the hyperglycaemic state and the progression to T2DM and CVD.

T2DM is characterised by hyperglycaemia primarily associated with insulin resistance but often obesity, dyslipidaemia, hypertension and accelerated atherosclerosis are clinical comorbidities (21). T2DM has been further classified as a chronic inflammatory state with evidence of disparate concentrations of cytokines and acute phase reactants (APR’s) (22). This inflammatory process and the association with T2DM is also contributory, but not necessarily exclusive to the
progression to CVD (23). More traditional inflammatory markers such as C-reactive protein (CRP), are recognised and utilised as a general high sensitivity systemic marker of inflammatory processes (24) and are of prognostic use in areas such as predicting coronary heart disease (25). However CRP lacks specificity for T2DM but in combination with other inflammatory and oxidative stress markers may improve risk prediction for T2DM and CVD as utilising current global risk assessment strategies and scores does still remain sub-optimal (26). The inflammatory markers interleukins-1β, 6, 10 (IL-1β, IL-6, IL-10), monocyte chemo-attractant protein-1 (MCP-1) and insulin like growth factor-1 (IGF-1) were explored in this study. Chronic low-grade inflammatory responses with activation of the innate immune system have been associated with diabetes, metabolic syndrome (MS) and atherosclerosis (27-29). Association between inflammation, oxidative stress and BGL have not been comprehensively investigated (30-32) and utilising these markers to classify participants attending a diabetes health screening clinic into those with possible preclinical CVD is by no means definitive. This study investigated the association of inflammatory and oxidative stress markers with normal BGL of <4.5mmol/L (1st quintile) and those with increased BGL >6.1mmol/L (4th-5th quintile).

Methods:

Three hundred and nine participants were recruited from the Diabetic Health Screening Clinic (DiabHealth) at Charles Sturt University. The study was approved by the Charles Sturt University Human Ethics Committee (Protocol Number 2006-042) and complies with the standards of the Helsinki agreement for human research. All patients were informed of the aims of the research and any risk prior to consent. As this was a screening clinic, patients were not discriminated or excluded based upon their medical and medication history. The control group (n =47) were extracted from attendees at the diabetic screening clinic who were non diabetic, normoglycaemic, normotensive with no evidence of CVD and were non-medicated. Anthropometric data was obtained (Table 1) in addition to the collection of blood and urine specimens. Specimens collected were analysed for blood glucose and lipids. Biomarkers for oxidative stress and inflammation were performed on all patient specimens. Body mass index (BMI) was measured using standardised beam weight scales. BMI is defined as weight in kilograms per height expressed as meters squared and is independent of gender and age. Waist
circumference (WC) was measured using a standard measuring tape. Measurements were taken between the top of the hip bone and lowest rib. Blood pressure was measured using a sphygmomanometer with appropriate cuff size after a 5-minute rest. The average of two measurements 1 minute apart was used for systolic and diastolic blood pressure values.

Specimen collection and processing:

Patients fasted overnight prior to data and blood collection. Whole blood specimens were collected into plain, heparin and EDTA anticoagulated, 7 mL tubes. Serum and plasma was separated after a 10 minute centrifugation at 1000g. Glucose, total cholesterol, triglycerides, HDL cholesterol, HbA1c and CRP were performed on the day of collection at the local pathology laboratory, in accordance with Australian Laboratory Standards. Plasma/serum/washed red blood cell (RBC) lysate samples required for rbcGSH, GSSG, 8OHdG and interleukins analysis were stored at -80°C prior to batch analysis.

General biochemistry:

At the screening clinic patients had a screening blood glucose performed and bloods collected prior to assessment. Plasma glucose, total cholesterol (TC), and triglycerides (TG) were determined by standard enzymatic kits and high-density lipoprotein (HDL) was determined using an immunoinhibition assay. Low-density lipoprotein cholesterol (LDL-C) was calculated according to the Friedewald formula. HbA1c and high sensitivity CRP were analysed by immunoassay. These investigations were carried out at Dorovitch laboratory, a national accredited private pathology laboratory.

Oxidative stress markers:

GSH and GSSG levels were measured from erythrocyte lysate by a Glutathione Assay Kit (Cayman Chemical, MI, USA). As the method incorporates glutathione reductase, total glutathione is measured. GSH reacts with Ellman’s reagent with the production of 5-thio-2-nitrobenzoic acid (TNB). Absorbance was measured at 405nm with the absorbance of TNB directly proportional to
the GSH concentration in the sample. The GSH:GSSG ratio was determined using the formula (total GSH-2GSSG)/GSSG. Results were calculated using a four parameter logistic fit.

Plasma from 8OHdG was assayed with an enzyme immunosorbent assay (EIA) Kit (Cayman Chemical, MI, USA). The test procedure utilises an anti-mouse IgG-coated plate and a tracer consisting of an 8OHDG-enzyme conjugate which detects all three oxidized guanine species; 8OHDG from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA. This kit analysis has the advantage of providing low variability and increased sensitivity compared with assays that utilise an antigen coated plate which only detect 8OHdG.

**Inflammatory markers:**

IL-1β, IL-6, IL-10, MCP-1 and IGF-1 levels were determined using Enzyme Linked Immunosorbent Assay (ELISA) kits (Elisakit.com, Adelaide Australia). The methods incorporated pre-coated IL, MCP & IGF capture antibody incubated with their appropriate anti-human (i.e. IL-1β, IL-6, IL-10, MCP-1, IGF-1) 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:**

Data analysis with descriptive data expressed as mean ± standard deviation (x ± SD), for demographic and anthropometric data and median ± IQR (interquartile range) for nonparametric data. A t-test was conducted on descriptive data. The statistical analysis for the 1st and 4th, 5th quintile was the rank based nonparametric Mann-Whitney test for a two group comparison. Data analysis was performed with PAWS (Version 22, IBM Co). Data was grouped by fasting blood glucose (BGL) <4.5mmol/L (1st quintile) vs BGL >6.1mmol/L (4th, 5th quintile) and compared, with results of p <0.05 considered significant.
Results:

The control group, who had no reported hypertension, CVD or diabetes as well as being medication free, is tabulated shown as a comparison to the clinic groups with results for the control group and 1st, 2nd, 3rd and 4th, 5th quintiles depicted in Table 1. Gender distribution was generally slightly higher for females than males. The study group were divided into quintiles. Statistical analysis of the 1st and 4th, 5th quintiles was based upon a fasting blood glucose (BGL) of Q1 (<4.5mmol/L) and Q4, 5 (>6.1mmol/L). Waist circumference and BMI were significant between groups ($p < 0.001$) as was the general biomarkers BGL, HbA1c, TG, HDL, TC/HDL ratio and AIP ($p < 0.001$).
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control group</th>
<th>Q1 (BGL ≤4.5 mmol/L)</th>
<th>Q2 (BGL ≥4.5-6.1mmol/L)</th>
<th>Q3 (BGL ≥6.1mmol/L)</th>
<th>Q4.5) BGL ≥6.1mmol/L</th>
<th>p value Q1 vs Q4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>20M/27F</td>
<td>19M/27F</td>
<td>16M/33F</td>
<td>30M/38F</td>
<td>49M/50F</td>
<td>ns</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>61.2 ± 9.9</td>
<td>69.8 ± 12</td>
<td>69.6 ± 8</td>
<td>66.6 ± 11</td>
<td>69.3 ± 9</td>
<td>ns</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>91.8 ± 12.5</td>
<td>91.8 ± 15.2*</td>
<td>93.6 ± 14.3</td>
<td>97.7 ± 10.8</td>
<td>104.2 ± 14.6*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.0 ± 4.6</td>
<td>25.9 ± 5.4*</td>
<td>27.6 ± 6.2</td>
<td>28.3 ± 4.4</td>
<td>29.9 ± 5.5*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>SBP (mm/Hg)</td>
<td>125.5 ± 15.2</td>
<td>130 ± 19</td>
<td>129.8 ± 17</td>
<td>132.8 ± 21</td>
<td>135 ± 19</td>
<td>ns</td>
</tr>
<tr>
<td>DBP (mm/Hg)</td>
<td>76.7 ± 7.4</td>
<td>74 ± 10</td>
<td>76 ± 8</td>
<td>79 ± 8</td>
<td>78 ± 19</td>
<td>ns</td>
</tr>
<tr>
<td>AIP</td>
<td>-0.18 ± 0.26</td>
<td>-0.19 ± 0.23*</td>
<td>-0.15 ± 0.26</td>
<td>-0.01 ± 0.25</td>
<td>0.08 ± 0.3*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>BGL (mmol/L)</td>
<td>5.2 ± 0.9</td>
<td>4.5 ± 0.3*</td>
<td>5.1 ± 0.1</td>
<td>5.6 ± 0.2</td>
<td>8.6 ± 3.2*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.6 ± 0.3</td>
<td>5.6 ± 0.5*</td>
<td>5.7 ± 0.3</td>
<td>5.7 ± 0.4</td>
<td>6.7 ± 1.3*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>5.3 ± 0.8</td>
<td>5.0 ± 0.9</td>
<td>5.3 ± 1.1</td>
<td>5.3 ± 1.0</td>
<td>4.7 ± 1.3</td>
<td>ns</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.2 ± 0.6</td>
<td>1.1 ± 0.5*</td>
<td>1.2 ± 0.5</td>
<td>1.5 ± 0.6</td>
<td>1.8 ± 0.9*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.6 ± 0.5</td>
<td>1.8 ± 0.5*</td>
<td>1.6 ± 0.5</td>
<td>1.5 ± 0.4</td>
<td>1.4 ± 0.5*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.22 ± 0.8</td>
<td>2.7 ± 0.9</td>
<td>3.1 ± 1.0</td>
<td>3.2 ± 0.9</td>
<td>2.6 ± 1.1</td>
<td>ns</td>
</tr>
<tr>
<td>TC/HDL ratio</td>
<td>3.4 ± 1.1</td>
<td>3.0 ± 0.9*</td>
<td>3.4 ± 1.2</td>
<td>3.8 ± 1.1</td>
<td>3.8 ± 1.3*</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Results expressed as mean ± SD. Q – quintile,* Statistical significance between Q1 - glucose of <4.5mmol/L and Q4, 5 glucose of >6.1mmol/L, ns – not significant, WC – waist circumference, BMI – Body mass index, SBP – systolic blood pressure, DBP – diastolic blood pressure, AIP – atherogenic index, BGL – blood glucose level, HbA1c – Haemoglobin A1c, TC – total cholesterol, TG – triglyceride, HDL – high density lipoprotein, LDL – low density lipoprotein

The comparison of oxidative stress and inflammatory markers (Table 2) compared the 1st and 4th 5th quintile. Significant results were observed for GSH (p <0.001), GSH:GSSG and 80HDG and the inflammatory marker IL-1β (p <0.05). In keeping with our previous publications GSH/GSSG results were not expressed with reference to Hb concentration. At examination, patients based upon haematocrit observations, were noted not to be anaemic or polycythaemic.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control Group</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q 4.5</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (µmol/L)</td>
<td>1760.2 ± 729</td>
<td>2267.8 ± 673*</td>
<td>1787.5 ± 791</td>
<td>1804.8 ± 1013</td>
<td>1632.2 ± 612*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GSSG (µmol/L)</td>
<td>286.4 ± 266</td>
<td>266.6 ± 110</td>
<td>275.5 ± 281</td>
<td>241.9 ± 100</td>
<td>352.4 ± 211</td>
<td>ns</td>
</tr>
<tr>
<td>GSH:GSSG ratio</td>
<td>5.8 ± 6</td>
<td>8.1 ± 4*</td>
<td>8.3 ± 10</td>
<td>7.4 ± 7</td>
<td>5.1 ± 5*</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>8-OHDG (pg/mL)</td>
<td>511.9 ± 264</td>
<td>612.5 ± 429*</td>
<td>647.6 ± 548</td>
<td>668.4 ± 398</td>
<td>865.2 ± 512*</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>2.85 ± 5</td>
<td>2.48 ± 1.5*</td>
<td>3.41 ± 11.7</td>
<td>2.75 ± 10.6</td>
<td>3.56 ± 8.5*</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>19.19 ± 21.9</td>
<td>15.76 ± 16.5</td>
<td>19.96 ± 29.3</td>
<td>15.72 ± 29.7</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>19.95 ± 21.1</td>
<td>28.1</td>
<td>17.45 ± 27.5</td>
<td>18.51 ± 25.1</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>MCP-1 (pg/mL)</td>
<td>196.95 ± 65.8</td>
<td>215.60 ± 102.3</td>
<td>189.02 ± 112</td>
<td>192.4 ± 139</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>IGF-1 (pg/mL)</td>
<td>295.58 ± 541.6</td>
<td>347.51 ± 447.4</td>
<td>307.38 ± 614.5</td>
<td>205.12 ± 362.1</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>1.0 ± 2</td>
<td>1.0 ± 1.9</td>
<td>1.0 ± 1.6</td>
<td>1.9 ± 2.2</td>
<td>1.8 ± 2.2</td>
<td>ns</td>
</tr>
</tbody>
</table>

Median ± IQR, Q - Quintile, * Statistical significance between Q1 and Q 4.5, ns – not significant, GSH – glutathione, GSSG – glutathione disulfide, 8-OHDG – 8-hydroxy-2-deoxyguanosine, IL-1β – interleukin 1 beta, IL-6 – interleukin 6, IL-10 – interleukin 10, MCP-1 – monocyte chemo-attractant protein -1, IGF-1 – insulin like growth factor – 1, CRP – C reactive protein
Figure 1. Medication usage chart comparison

![Image of medication usage chart comparison]

Dmeds – diabetic medication, Anti HT – antihypertensives, NSAID – non steroidal anti-inflammatory drugs

Figure 1 expresses medicated patients with each medication classification portrayed as a percentage of the total for the respective glucose level.

Inflammatory markers were expressed as ratios (Table 3) and the 1st and 4th, 5th quintiles were compared. IL-6/IL-10, IL-1β/IL-10, MCP-1/IL-10, IGF-1/IL-10 and IL-6/IL-1β were significant (p <0.05)

<table>
<thead>
<tr>
<th></th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4,5*</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP/IL-10</td>
<td>0.12 ± 0.4</td>
<td>0.08 ± 0.1</td>
<td>0.09 ± 0.4</td>
<td>0.06 ± 0.2</td>
<td>ns</td>
</tr>
<tr>
<td>IL-6/IL-10</td>
<td>0.26 ± 0.4*</td>
<td>0.41 ± 2.3</td>
<td>0.53 ± 0.5</td>
<td>0.44 ± 1.2*</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>IL-1β/IL-10</td>
<td>0.17 ± 0.1*</td>
<td>0.34 ± 1.0</td>
<td>0.19 ± 0.5</td>
<td>0.32 ± 0.6*</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>MCP-1/IL-10</td>
<td>9.4 ± 11.2*</td>
<td>10.93 ± 14.0</td>
<td>8.90 ± 17.3</td>
<td>8.65 ± 14.3*</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>IGF-1/IL-10</td>
<td>15.4 ± 20.1*</td>
<td>16.31 ± 17.0</td>
<td>7.84 ± 8.1</td>
<td>8.13 ± 22.9*</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>IGF-1/MCP-1</td>
<td>1.52 ± 1.7</td>
<td>1.29 ± 1.7</td>
<td>0.96 ± 1.8</td>
<td>0.99 ± 3.2</td>
<td>ns</td>
</tr>
<tr>
<td>IL-1β/CRP</td>
<td>1.59 ± 2.3</td>
<td>7.4 ± 10.9</td>
<td>2.05 ± 5.6</td>
<td>2.78 ± 12.2</td>
<td>ns</td>
</tr>
<tr>
<td>IL-6/IL-1β</td>
<td>1.87 ± 1.9*</td>
<td>1.07 ± 18.6</td>
<td>2.54 ± 12.3</td>
<td>3.05 ± 13.4*</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>CRP/IL-6</td>
<td>0.50 ± 0.9</td>
<td>0.30 ± 0.3</td>
<td>0.53 ± 0.8</td>
<td>0.28 ± 0.8</td>
<td>ns</td>
</tr>
</tbody>
</table>
Median ± IQR, Q – quintile, Statistical significance Q1 vs Q4,5, CRP – C reactive protein, IL-10 – interleukin-10, IL-6 – interleukin-6, IL-1β – interleukin-1 beta, MCP-1 – monocyte chemo-attractant protein-1, IGF-1 – insulin like growth factor -1, ns – not significant.

**Figure 2.** CVD, HT and T2DM status

T2DM – Type 2 diabetes mellitus, CVD – cardiovascular disease, HT - hypertension

Figure 2 - Patients selected for this study attended the Charles Sturt University rural health diabetic screening program and were not discriminated on the basis of clinical or medication status. The results express the number of CVD, HT and T2DM patients as a percentage of the total for each respective group. The CVD, HT and T2DM group is the combined sum of patients with all 3 categories present.

**Discussion**

This study highlights the potential value emerging inflammatory and oxidative stress biomarkers may contribute to the screening of patients that exhibit a hyperglycaemic state irrespective of existing treatment regimens targeting T2DM, CVD and HT. Our results have replicated findings of well-controlled experiments comparing oxidative stress and inflammatory markers in diabetes progression, and demonstrated a substantive association with hyperglycaemia, oxidative stress and the inflammatory in a screening population (10, 33-36). The impact of oxidative stress and the relationship with the inflammatory response was further supported upon a review of the clinical status indicating a higher prevalence of self-reported CVD and hypertension as shown in
Figure 2 most likely due to T2DM, CVD and HT being associated with inflammatory responses and oxidative stress (18, 33, 37, 38).

Hyperglycaemia is associated with increased risk of T2DM associated with oxidative stress and an inflammatory process (19). Significant differences were observed in WC, BMI, BGLs, HbA1c, TG, HDL and TC/HDL between the normoglycaemic and elevated glycaemia groups (Table 1). These findings support their association with T2DM and affirms that traditional markers do assist to some extent in demonstrating that a continuum exists between chronic increasing hyperglycaemia and the progression to and development of T2DM. HbA1c values of 6 – 6.5% may represent a high risk for the development of diabetes (if used for diagnostic purposes), however there is still a continuum of risk associated with normal levels of HbA1c below 6% (39). If a lower cut-off of HbA1c is used then the likelihood of missing DM is also increased.

The constellation of oxidative stress and inflammation factors emerging from and participating with the pro inflammatory hyperglycaemic state is not fully understood, but of major concern is the progression of patients with elevated glucose to T2DM and the associated morbidity (27, 30, 40). The results of this study indicate that both oxidative stress and inflammation provided significant results when groups were categorised with a BGL <4.5mmol/L and > 6.1mmol/L (Table 2), irrespective of any medication. The oxidative stress markers GSH, GSH:GSSG and 8OHdG showed significant results. GSH has previously been shown to demonstrate an association with IFG and oxidative stress and the link to diabetes (7, 41, 42). It has also been demonstrated that GSH:GSSG showed a significant difference between a control group and impaired fasting glucose (IFG), supporting the notion that early thiol related oxidative stress occurs (14). If this is the case, and as demonstrated in this study, the significant GSH, GSH:GSSG results when glucose levels were >6.1mmol/L, support the concept of monitoring oxidative stress during sustained hyperglycaemia. A consequence of this is an increased risk of micro and macrovascular complications (43) due to the increased ROS contributing to the structural and functional damage of endothelial cells and smooth muscle proliferation, a factor in the development of atherogenesis (44, 45). 8OHdG is also considered an important biomarker for oxidative stress in diabetes progression and indicator of the pre-diabetic state (7). There is still some controversy over whether oxidative stress is triggered by sudden onset hyperglycaemia or at least pronounced glucose variability (46), however there is merit in the continuous monitoring.
of BGL along with oxidative stress biomarkers and antioxidant status for diabetes progression and complications screening. This would further aid in establishing whether a patient is at risk of developing T2DM or its complications based on the additional information obtained for oxidative stress and inflammation.

It has also been demonstrated that there are positive and negative oxidative stress associations, which have been linked to lipid levels (47-49). In this study lipid studies did show a significant difference between glucose levels <4.5mmol/L and levels >6.1mmol/L. As we did not discriminate between disease and the medication state, the oxidative stress markers provide an important additional diagnostic tool, along with inflammatory markers in their utility in screening and interpreting possible diabetes risk in mild to moderate hyperglycaemic patients.

Elevated levels of the pro-inflammatory IL-1β has been associated with hyperglycaemia, insulin resistance, obesity, all factors which contribute to the development of T2DM (50). The current study confirmed that a significant increase of IL-1β is associated with hyperglycaemia and supports previous findings of elevated IL-1β in T2DM, possibly as a result of chronic inflammation and insulin resistance (51). Studies have shown that the neutralization of IL-1β assists in decreasing the inflammatory response in autoimmune, malignancy and other common disease states such as T2DM (52, 53). If so knowledge of IL-1β levels may provide an indication of earlier treatment intervention to allay the development of progression to T2DM and CVD in hyperglycaemia. Elevated IL-6, another pro-inflammatory marker, has also been demonstrated in hyperglycaemia/hyperinsulinaemia (54, 55) and in a previous study of ours (33). The anti-inflammatory marker IL-10 in this study was not significantly different between the two glucose groups. IL-10 is considered a potent anti-inflammatory, however mixed results have been observed (56). Esposito has pointed out that a low innate production capacity of IL-10 may help identify subjects at more risk for inflammation in MetS (27, 57). Drug therapy to either control T2DM or prevent development of comorbidities is required if diet and lifestyle improvements are unable to modify and improve the risk factors resulting from hyperglycaemia. Whilst the anti DM, anti HT and statin use is much higher in the elevated glucose group (>6.1mmol/L), the use of NSAIDS was also heavily utilised in the low glucose (<4.5mmol/L) group (Fig 1). This may exert an effect on the anti-inflammatory effects of IL-10, however it has been shown that statins not only reduce cholesterol and TG but can independently decrease CRP levels (58).
Recognising in this study that IL-1β was the only individual inflammatory marker demonstrating a significant difference, a ratio test of the emerging biomarkers, with CRP included, was investigated for any degree of significance between the Q1 and Q4, 5 groups (Table 3). Previous studies into prognostic inflammatory outcomes has revealed an association of IL6/IL10 in systemic inflammatory responses and post-operative surgical outcomes to infections in chronic alcoholics (59, 60). Studies on inflammatory markers have noted that participants with a combined elevation of both IL-6 and IL-1β had about a three-fold increase in risk of developing diabetes, whereas low levels of IL-1β alone demonstrated no substantial increase in risk (32). However to our knowledge, inflammatory marker ratios and any association with hyperglycaemia, has not been fully investigated, albeit the recognition that there are numerous possible interactions between inflammatory markers and the presence of T2DM (61, 62). A greater understanding of the interactions via ratio testing may therefore provide valuable information in T2DM and CVD progression. Significant results were obtained with IL-6/IL-10, IL-1β/IL-10, MCP-1/IL-10, IGF-1/IL-10 and IL-6/IL-1β (Table 3). Studies reviewing IL-6 and tumor necrosis factor-α (TNF-α) have established roles in the regulation of APR’s with other investigations providing a possible link with these two biomarkers and cardiovascular events (63). Additionally an association with TNF-α receptor 2, IL-6 and CRP has been demonstrated, with elevated levels of CRP noted as a strong independent predictor of T2DM. CRP as a possible mediator between TNF-α receptor 2 and IL-6 IL-6/IL-10 has been shown to decrease significantly in post-operative alcoholic patients with infections (60). It has been previously established that the anti-inflammatory IL-10 is induced by pro-inflammatory cytokines such as IL-6, thereby affording some protection in states of inflammation (64). There is also growing evidence linking lower levels of IL-10, independently of increased levels of IL-1β, with obesity, MetS and CVD (65). Our results show that IL-1β/IL-10 was significantly different between the low BGL versus high BGL group, whereas IL-10 did not increase significantly compared to the control group suggesting that IL-10 is not responding to small increases in BGL. This could also reflect possible interactions between IL-6, MCP-1, or IGF-1, whether acting dependently or independently of IL-10, with the ratio providing a more sensitive indicator in the development and pathophysiology of T2DM and CVD. The pro-inflammatory IL-6/IL-1β ratio was significantly higher where the individual analysis of the inflammatory markers other than IL-1β was not; possibly indicating the requirement for caution in the use of individual biomarkers as a
screening mechanism when investigating T2DM and CVD. IL-1β has been shown to be increased in T2DM (35), however its interaction with functional β-cell mass leading to overt T2DM may not be reversible by glucose lowering therapy (66), thus further highlighting that inflammatory cytokine and oxidative stress ratios may provide additional information on progressive hyperglycaemia in a screening cohort and their potential use as clinical indicators of T2DM and CVD disease progression and targets for therapy.

**Conclusion**

Our current results support previous findings that inflammation and oxidative stress are associated with increased blood glucose levels and suggest that GSH, GSH:GSSG, 8O HdG and IL-1β may play a role in aiding clinicians in identifying hyperglycaemic patients with inflammatory and oxidative stress pathology and are at risk of developing T2DM and CVD. Our novel findings observed with the inflammatory marker ratios have not been previously reported. Using anthropomorphic, traditional markers and emerging marker’s and/or ratios in hyperglycaemic management may provide valuable information leading to more preventative measures at a clinical level. As these novel ratio findings are largely untested in the hyperglycaemic state further investigations are required to interrogate our findings. Future research will expand this analysis on inflammatory and oxidative stress markers and whether they can assist in characterising diabetes and its progression from a prediabetic (IFG) level.

**Conflict of Interest**

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article

**Acknowledgement**

Roche Australia provided the glucose measuring sticks and glucometers. Bev de Jong provided technical assistance Simon McDonald from the CSU Spatial Data Analysis Network assisted in the statistical analysis.
References


Appendix H: Antidiabetic, antihypertensive and statin medication use in metabolic syndrome

Introduction

The metabolic syndrome (MetS) is a cluster of metabolic risk factors associated with a 5-fold increased risk of type 2 diabetes (T2DM) and a 2-fold increased risk of atherosclerotic cardiovascular disease. The National Cholesterol Education Program Adult Treatment Panel III (ATPIII) has defined a set of criteria to identify patients having the MetS and viewing CVD as the primary clinical outcome of this disease [1,2]. The 5 criteria identified by the ATPIII of which the presence of any three or more comprise the MetS is listed in Table 1 [3].

Table 1: ATPIII Modified Clinical Identification of the Metabolic Syndrome.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Defining Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waist circumference (cm)</td>
<td>≥ 102</td>
</tr>
<tr>
<td>Men</td>
<td>&gt; 85</td>
</tr>
<tr>
<td>Women</td>
<td>&gt; 85</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>≥ 1.7</td>
</tr>
<tr>
<td>Men</td>
<td>&lt; 1.04</td>
</tr>
<tr>
<td>Women</td>
<td>&lt; 1.30</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>≥ 1.04</td>
</tr>
<tr>
<td>Men</td>
<td>&lt; 1.04</td>
</tr>
<tr>
<td>Women</td>
<td>&lt; 1.30</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>≥ 130 ≥ 85</td>
</tr>
<tr>
<td>Fasting Blood Glucose (mmol/L)</td>
<td>≥ 6.1</td>
</tr>
<tr>
<td>Use of anti-diabetic, antihypertensive or Statin medication</td>
<td>*HDL = high density lipoprotein.</td>
</tr>
</tbody>
</table>

Whilst insulin resistance is not a required criterion for MetS using the ATPIII classification, the presence of T2DM or antihyperglycemic medication is considered in its diagnosis [3]. Additional definitions have been recommended by the World Health Organization (WHO), American Association of Clinical Endocrinologists and the International Diabetes Federation (IDF) [4,5]. Whilst these are some important differences in ranking of the predominant causative factors, there is recognition of similar criteria to the ATPIII definition of MetS. However, a major difference between the definition of the ATPIII and the IDF is the latter does include the patient’s medication as a criterion for the MetS. This additional criterion does allow either BGL or triglycerides to be in the normal range.

One of the most important risk factors leading to T2DM is the presence of prediabetes. Prediabetes is defined by either an impaired fasting (BGL > 6.1mmol/L) or post-prandial blood glucose level (BGL > 11mmol/L). Together with other potential risk factors for CVD, according to the ATPIII classification prediabetes is a major cause of the metabolic syndrome and one of its defining factors [6]. Additional underlying metabolic risk factors such as obesity and abnormal body fat distribution account for 20% and 30% of the adult population and predispose to MetS [7,8]. Although not included in the ATPIII classification, age correlates positively with MetS [9].

MetS and glucose lowering medication

A common finding and independent diagnostic criterion for
MetS is the presence of hyperglycaemia. Whilst the major studies conducted in 2008 and 2009 - UK Prospective Diabetes Study (UKPDS), Veteran’s Affairs Diabetes Trial (VADT), Action to Control Cardiovascular Risk in Diabetes (ACCORD), Action in Diabetes and Vascular Disease (ADVANCE) and Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycaemia in Diabetes (RECORD) reviewed the extensive use of antihyperglycaemic agents as primary strategies in the treatment for MetS, the need for more intensive glycaemic control may also provide cardiovascular benefit for early T2DM with no demonstrated presence of atherosclerosis [10]. If insulin resistance is one of the risk factor in MetS, improving glucose control and insulin resistance through pharmaceutical agents will be another target to be considered in addition to physical activity. However, some drug strategies such as use of metformin for improving insulin resistance are not routinely used for decreasing risk of T2DM and CVD if prediabetes is present [4]. Mixed results have been reported on whether antihyperglycaemic medication decreases CVD risk in patients with prediabetes or T2DM. Only empagliflozin has been shown to improve cardiovascular prognosis. In a recent prospective study, cardiovascular events have not increased with insulin treatment with or without metformin [11,12].

Antihypertensive medication and MetS

Antihypertensive drug treatment is recommended for MetS patients when BP is >140/90mmHg. Observations from the Framingham Risk Study (FRS) state that vascular disorders are central to MetS as indicated that 80% of men and up to 65% of women with hypertension are obese [13]. Insulin resistance has been associated with the development of HT, possibly through a variety of mechanisms involving sodium imbalance, imbalance between the release of nitrous oxide and endothelin-1, insulin action, adipokine activity due to increased adipose tissue and obesity (including perivascular adipose tissue and vascular function), decreased levels of adiponectin, adipokine activity and increased tumour necrosis factor α (TNF-α) [14]. Additionally, the importance of genetics cannot be underestimated. Hopkins and Huns (2003), have provided an extensive review of genetic markers that may contribute to the development of HT [15]. Whilst genetic analysis is still somewhat impractical and economically prohibitive as a diagnostic screening tool, as technology continues to improve these costs will come down.

MetS and statins

The statins are a class of drugs which act to lower total cholesterol and LDL levels by reducing hepatic cholesterol production through inhibition of hydroxyl methyl glutaryl Co-A (HMG-CoA) reductase and a reduced CVD incidence [16]. Statins are also known to reduce circulating triglyceride levels [17]. 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 [18]. As MetS may progress to T2DM and increased CVD it should be considered to be an inflammatory state. The use of statins has been shown to decrease circulating levels of C Reactive protein (CRP), independently to its lipid lowering effect [19].

The successful treatment of MetS involves addressing all of the risk factors treatment regimes. Whilst lifestyle and diet has emerged as a major preventative approach, these changes alone may not control or prevent the development of the risk factors categorising MetS. The current study investigated the use of antihyperglycaemic, antihypertensive and lipid lowering (statins) drugs and their associated use in MetS and how medication use differs with respect to the number of MetS factors identified.

Materials and Methods

Data for this study was obtained from patients attending a diabetes health screening clinic (DiabHealth) in south-eastern Australia between 2005 and 2011. Participants were recruited via public media announcements. The screening and data collection were carried out within the School of Community Health at Charles Sturt University (CSU). Participants had a medical history taken and anthropometric data collected in addition to screening for MetS factors. Thresholds for MetS criteria were taken from the definition of the National Cholesterol Education Program Adult Treatment Panel III (ATP III) (Table 1). Participants who met three or more criteria were classified as MetS positive. In the current study, medication use was also taken into account in classifying patients into the No MetS or MetS group as described in the definition of the International Diabetes Federation (IDF).

Age, gender, body mass index (BMI) (low <20 kg/m², normal <25 kg/m², overweight 25–30 kg/m², and obese >30 kg/m²) and waist circumference (measured at the midpoint between the lower border of the rib cage and the iliac crest by using a flexible inch tape) were obtained. Blood pressure (BP) measurements were taken using a standard mercury sphygmomanometer and a cuff of appropriate size after the individual had rested for at least five minutes in a supine position. BP was recorded in a sitting position in five individuals with the arm supported at heart height, as this was more comfortable for these five patients. A comprehensive list of prescription medications was provided by each patient. Medication profile for each participant was collected and data sorted into antihyperglycaemic, statin and antidiabetic use.

The data was analysed using R statistical computing (Version 3.2.3 for Windows) and Microsoft Excel (Office2007, Microsoft). All values were expressed as mean ± standard deviation (M±SD). Statistical analysis was performed using an independent sample t-test for two group comparisons of continuous normally distributed data or Chi-square statistics were used to investigate categorical data. In addition proportions analysis was used to compare the data between the five MetS factors. Post-hoc pairwise comparisons were performed using the Benjamini and Yekutieli correction after significant effects were found following the proportions test [20]. ANOVA and post hoc statistics was applied for linical continuous multigroup data. In all tests, p < 0.05 was considered to be statistically significant. Power analysis was based on a median effect size and high power, suggesting a sample number of 27 with a p value of 0.05 to be sufficient to establish meaningful differences [21].

Results

During the screening period from January 2005 to October 2011, 1614 volunteers attended the Diabetes Health (DiabHealth) clinic
Table 2: ATP III factors of the study population.

<table>
<thead>
<tr>
<th></th>
<th>MetS</th>
<th>No MetS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC, Females*</td>
<td>98.8 ± 11.6</td>
<td>84 ± 14.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>WC, Males</td>
<td>101.5 ± 13.2</td>
<td>103.8 ± 11.7</td>
<td>ns</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>120.7 ± 17.3</td>
<td>131.5 ± 17.4</td>
<td>ns</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>76.1 ± 8.6</td>
<td>76.3 ± 5.1</td>
<td>ns</td>
</tr>
<tr>
<td>HDL, Females</td>
<td>1.54 ± 0.4</td>
<td>1.5 ± 0.4</td>
<td>ns</td>
</tr>
<tr>
<td>HDL, Males</td>
<td>1.33 x ± 0.6</td>
<td>1.2 ± 0.3</td>
<td>ns</td>
</tr>
<tr>
<td>Triglycerides (mMol/L)</td>
<td>1.47 ± 0.9</td>
<td>1.42 ± 0.8</td>
<td>ns</td>
</tr>
<tr>
<td>BGL (mMol/L)</td>
<td>5.5 ± 1.7</td>
<td>5.5 ± 1.8</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*WC: Waist Circumference; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; HDL: High Density Lipoprotein; BGL: Blood Glucose Levels; mean ± standard deviation; ns = non significant.

Table 3: Percentage use of medication for patients with and without metabolic syndrome.

<table>
<thead>
<tr>
<th>Medication</th>
<th>MetS (%)</th>
<th>No MetS (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Diabetics</td>
<td>51.21%</td>
<td>7.2%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Anti-Hypertensions</td>
<td>62.87%</td>
<td>78.6%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Statins</td>
<td>52.21%</td>
<td>26.8%</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*MetS: Metabolic Syndrome present (Factors ≥ 3); No MetS: Metabolic Syndrome not present (Factors < 3); Percentage.

Table 4: Number of patients using diabetes medication for each MetS factor.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Diabetics*</th>
<th>Total N</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>100</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>116</td>
<td>5.2</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>132</td>
<td>15.9</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>90</td>
<td>22.5</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>27</td>
<td>44.4</td>
</tr>
</tbody>
</table>

*Diabetics: number of patients on one or more antidiabetic medication.

Table 5: Number of participants using antihypertension medication for each MetS factor.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Anti-HT*</th>
<th>Total N</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>105</td>
<td>26.6</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>116</td>
<td>46.6</td>
</tr>
<tr>
<td>3</td>
<td>81</td>
<td>132</td>
<td>61.4</td>
</tr>
<tr>
<td>4</td>
<td>59</td>
<td>80</td>
<td>73.8</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>27</td>
<td>81.5</td>
</tr>
</tbody>
</table>

*Anti-HT = antihypertensive medication.

Table 6: Number of participants using statins for each MetS factor.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Statins</th>
<th>Total N</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>106</td>
<td>3.8</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>116</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>132</td>
<td>21.2</td>
</tr>
<tr>
<td>4</td>
<td>19</td>
<td>80</td>
<td>23.8</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>27</td>
<td>18.5</td>
</tr>
</tbody>
</table>

*W: Percentage.

Participants were screened for their medication in context with MetS. Groups were divided into no MetS (0–2 factors) and MetS (3–5 factors). Of 531 patients attending the Diab Health screening 70 were clear of any MetS factors and were receiving no antidiabetic, antihypertensive or statin medication.

Antidiabetic, antihypertensive and Statins use combined differed significantly between the MetS and No MetS groups (p < 0.001). When medication use was separated into anti-diabetic, antihypertensive and Statins, similar significant differences was found between the MetS and No MetS groups (Table 3).

In the following Tables (Tables 4–6) medication use with respect to presence of MetS factors is shown for antidiabetes (Diabetes) and antihypertensive (anti-HT) medication and Statins.

Antidiabetic medication use tripled (p < 0.001) when going from No MetS (< 3 factors) to MetS (≥ 3 factors). Comparing medication use with respect to number of ATP III factors present indicated significant differences (p < 0.001) between medication use and the number of ATP III factors present except between 1 and 2, 2 and 3, 3 and 4, and 4 and 5 factors present (Table 4).

Anti-hypertensive medication also increased significantly with the number of factors considered (p < 0.001). However no significant increases were noted when the number of ATP III factors increased from 3 to 5 (Table 5).

Statin usage increased with the number of MetS factors present. Significant differences in statin use with respect to number of factors present (Table 6) was seen for all comparisons (p < 0.001) except when comparing between 2 and 3, 3 and 4 and between 4 and 5 factors present (Table 6).

Comparison of antidiabetic agents, antihypertensive medication and Statins in Figure 1 indicates that the antihypertensive class is more prescribed in association with all categorizes. In general statin use is less prescribed in this population than diabetes in the MetS group (≥ 3 factors). The use of antidiabetic agents steadily increases and is similar to the antihypertensives once five factors of MetS are present in the patients.

Effectiveness of treatment with respect to MetS factors is shown for the MetS factors indicated by ATP III (Table 1).

The only significant difference observed was for waist circumference in females when between 1 and 3 MetS factors were
present (p<0.01). However, waist circumference for females was at best borderline, observed when 3 ATPIII factors were present and was highest for the group with one MetS factor. Similarly, waist circumference for males was only within the desired level if three or four ATPIII factors were present. All other differences in biomarker levels associated with the number of ATPIII factors present showed no significant differences. Triglyceride levels for 5 factors was above the desirable level of <1.7mmol/L [6]. For HDL, the desirable levels for females are >1.3 mmol/L and for males >0.4 mmol/L. Only the group with any one of the ATPIII factors present had an elevated mean BGL value (Table 7).

Discussion

ATP III criteria for diagnosis of MetS are practical to use in a clinical setting. According to ATPIII the presence of any three factors (Table 1), constitutes MetS [23]. Management of MetS must first start by addressing factors that are modifiable such as smoking, alcohol use and lack of physical exercise. Prophylactic use of medications such as Statins may also be warranted even on patients with normal cholesterol levels suggested by outcomes from the Heart Protection Study Collaboration (HPS) and the Collaborative Atorvastatin Diabetes Study (CARDS) [24,25]. The most widely recognised of the metabolic risk factors associated with metabolic syndrome are high cholesterol, hypertension, and elevated blood glucose levels. Depending on which factors are present MetS increases the risk of overt diabetes and cardiovascular disease [26]. Drug therapy is essential if modifiable risk factors such as lifestyle practices and diet are not controlling abnormal levels of BGL, systolic blood pressure and cholesterol. The current study investigated the use of medication reported by patients attending a diabetes health screening clinic (DiabHealth) and the presence of single and multiple MetS factors. The study classified MetS as the presence of any three factors of five present as defined by the ATPIII classification system but included the use of medication for raised BGL, blood pressure and LDL as an additional criterion as suggested by the IDF classification.

The biggest increase of medication when comparing MetS to NonMetS (<3 factors vs ≥3 factors) was seen for antidiabetic medication use suggesting that incidence of diabetes may be strongly related to the increase in obesity, blood pressure and cholesterol levels as observed in this study where the mean waist circumference was elevated in the nonMetS group and remained borderline in the MetS group. BGL was significantly different between the two groups but was lower in the MetS group possibly associated with the increase in patients with T2DM and the associated use of antidiabetic medication in the MetS group (Table 3) [23]. Analysis of medication use with respect to the number of MetS factors present indicated that there was no significant increase between any of the biomarkers. Antidiabetic medication use tended between 2 and 3 MetS factors present and the most significant difference was observed between one and five MetS factors present. This reflects the importance of dealing with hyperglycaemia following the Insulin Resistance Atherosclerosis Study (IRAS), which reported a nearly five times greater risk of coronary artery disease for the group with the lowest insulin sensitivity [1].

Antihypertensive medication was the most often prescribed group (31.1%) compared to the antidiabetic (6.4%) and Statins (19.5%) if only 1 MetS factor of the possible five was present (Table 1). SBP was borderline as recommended by ATPIII for factors 0 to 3. A dramatic increase in the mean of SBP to above 140mmHg was seen in association with 4 MetS factors present, which then dropped to ideal levels when 5 factors were present.

Low to moderate-dose statins is the recommended medication therapy for middle-aged patients with a CVD risk of above 10%. For patients with a lower CVD risk statins should be offered selectively and consider patient preference [27]. The current study found that there was a dramatic rise in statin use when the number of MetS risk factors increased from 1 to 2 but then remained steady with a decrease back to the level found with 2 MetS factors present when 5 factors were present. This result reflects the biomarker levels reported with no significant difference between the No MetS group and MetS group for CVD risk factors apart from waist circumference (p < 0.001), which decreased significantly below the MetS cut-off in the MetS group. The cholesterol biomarkers were all within normal limits. Disparities in our study with medication use are associated with our non-specific categorisation of the MetS characteristics where the presence of any one factor can be any one of the five and the presence of three or more the combination of any of the five factors defined by ATPIII. For Table 7, indicates that only waist circumference is above the cut-off value recommended by ATPIII. However SBP and cholesterol levels are below the cut-off due to the use of antihypertensive and statin use. This suggests that preventative measures are having an effect on preclinical MetS (<3 factors present) and BGL, blood pressure and total cholesterol and HDL are controlled in the MetS patient group, which show levels lower than those found in the non-MetS group. Medication use increases with an increase in ATPIII factors present in the study. However, participants with increased BGL (>6.1mmol/L) were not found to have antihyperglycaemic medication prescribed. Both antihypertensive medication and statins were extensively prescribed in cases where only 1 and 2 ATPIII factors for MetS were present. Several limitations of the study have to be noted including the self-reporting of medication use and the associated compliance by participants is not verified. In addition confounding factors may play a role in medication use, especially in the non-MetS group who may have only one or two MetS factors present such as economic status and education level.
Table 7: ATP III biomarker levels with respect to number of MS factors.

<table>
<thead>
<tr>
<th>Factors</th>
<th>WCF (mmHg)</th>
<th>WCM (mmHg)</th>
<th>Triglycerides (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>SBP (mmHg)</th>
<th>BGL (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98±11.5</td>
<td>104.1±13.3</td>
<td>1.41±0.7</td>
<td>1.6±0.4</td>
<td>1.55±0.4</td>
<td>130.9±17.6</td>
<td>6.3±2.2</td>
</tr>
<tr>
<td>2</td>
<td>82.4±16.6</td>
<td>103.4±11.3</td>
<td>1.41±0.9</td>
<td>1.46±0.4</td>
<td>1.47±0.4</td>
<td>131.7±17.4</td>
<td>5.6±1.5</td>
</tr>
<tr>
<td>3</td>
<td>88.5±12.7</td>
<td>101.3±13.3</td>
<td>1.65±1</td>
<td>1.52±0.4</td>
<td>1.51±0.4</td>
<td>131.1±17.7</td>
<td>5.6±1.7</td>
</tr>
<tr>
<td>4</td>
<td>89.4±14.4</td>
<td>98.2±12.7</td>
<td>1.28±0.6</td>
<td>1.6±0.5</td>
<td>1.65±0.4</td>
<td>124.3±14.5</td>
<td>5±1.1</td>
</tr>
<tr>
<td>5</td>
<td>91.3±14.9</td>
<td>111.8±18.7</td>
<td>2.1±1.6</td>
<td>1.58±0.2</td>
<td>1.47±0.3</td>
<td>142.3±11.7</td>
<td>5±2.6</td>
</tr>
</tbody>
</table>


Findings in our study indicates that in the focused community of outpatients the metabolic syndrome is relatively well controlled and the majority of the risk factors for CVD are below the documented threshold level. However, waist circumference remains higher than recommended, suggesting that lifestyle interventions may need to be addressed more to achieve an optimum response to the treatment [28]. Statin use may also be below that recommended as the increased SBP and antihypertensive medication use category is quite high (Figure 1) suggesting that there is a high risk of CVD in this population.

Limitation

The South-eastern Australian area has a diverse multicultural population. This study did not discriminate on the basis of ethnicity and therefore further investigations accounting for race may provide additional information.

Acknowledgement

Roche Australia provided the glucose measuring sticks and glucometers. Dev Long provided technical support.

References


Appendix I: Interaction of homocysteine, glutathione and 8-hydroxy-2’- deoxyguanosine in metabolic syndrome progression

Interaction of homocysteine, glutathione and 8-hydroxy-2’-deoxyguanosine in metabolic syndrome progression

Butkowski, E.G.¹ Al-Aubaidy, H.A.² and Jelinek, H.F.¹,³*

¹ School of Community Health, Charles Sturt University, Albury, Australia.
² School of Medicine, University of Tasmania, Hobart, Australia.
³ Australian School of Advanced Medicine, Macquarie University, Sydney, Australia.

*Corresponding author.
Herbert Jelinek
School of Community Health
Charles Sturt University
Albury,
Australia
T: +61 2 60519219
E: hjelinek@csu.edu.au
Abstract

Purpose: The role of homocysteine (Hcy) and associated oxidative stress processes in the metabolic syndrome (MetS) continuum has not been explored extensively. This paper studies changes in Hcy and associated oxidative stress in relation to the number of metabolic syndrome factors present.

Method: Participants (n = 266) attending a rural diabetes screening clinic had their medical history recorded as well as Hcy, body mass index, blood glucose levels, cholesterol, glutathione (GSH), and 8-hydroxy-2-deoxyguanosine (8-OHdG) measured.

Result: A significant elevation in Hcy (9.5 µmol/L ± 2 vs. 10.6 µmol/L ± 3, p = 0.03) and 8-OHdG (307 pg/mL ± 516 vs. 1130 pg/mL ± 1155, p = 0.0001) was observed between the noMetS and MetS groups. Hcy steadily increased with the addition of MetS factors paralleled by 8-OHdG and GSH. The most dramatic increase was seen in 8-OHdG, which nearly doubled between 2 MetS and 3 MetS factors present (p = 0.0001).

Conclusion: Homocysteine may be a useful marker together with 8-OHdG in assessing the extent of metabolic syndrome in a clinical rural population.

Keywords: Homocysteine, 8-OHdG, glutathione, metabolic syndrome, oxidative stress,

Abbreviations:
BMI – Body Mass Index
CRP – C - reactive protein
CVD – cardiovascular disease
DBP – Diastolic blood pressure
GSH – Glutathione
GSSG – Glutathione disulfide
HbA1c – Glycated hemoglobin
Hcy - Homocysteine
HDL – High density lipoprotein
IGF-1 - Insulin-like growth factor-1
IL – Interleukin
LDL – Low density lipoprotein
MCP-1 - Monocyte Chemotactic Protein-1
MDA – Malondialdehyde
MetHb - Methemoglobin
MetS – Metabolic syndrome
8-OHdG – 8-hydroxy-2’-deoxyguanosine
PGI2 – Prostaglandin-I2
T2DM – Type 2 diabetes mellitus
TC – Total cholesterol
TNF-α – Tumor necrosis factor-α
TG – Triglycerides
SBP – Systolic blood pressure
WC – Waist circumference
Introduction

Metabolic syndrome can be defined by a clustering of fasting hyperglycemia, decreased HDL-Cholesterol, hypertriglyceridemia, increased waist circumference (WC) and hypertension (HT) according to the National Cholesterol Education Program (NCEP) and Adult Treatment Program III (ATP III) (1). These defined clinical parameters are used to identify patients having MetS and view cardiovascular disease (CVD) as the primary clinical outcome of this disease (2, 3). Table 1 depicts the ATPIII criteria of which the presence of any three or more comprise the MetS (4). The clustering of MetS factors and the associated 5 fold increased risk of diabetes (T2DM) and 2-fold risk of atherosclerotic CVD are a major concern for global health (5, 6).

A major shortcoming of current definitions of MetS is the lack of inclusion of measures of a proinflammatory state and oxidative stress (7) as several inflammatory markers have been identified that may provide additional clinical information about MetS and disease progression including c-reactive protein (CRP), malondialdehyde (MDA), prostacyclin (PGI₂), interleukin - 6 (IL-6) and tumour necrosis factor – α (TNF-α) (8, 9). Emerging biomarkers of oxidative stress, inflammation and endothelial dysfunction have also been linked in the development of diabetes (10-13).

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Defining Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Criteria 1: Waist circumference (cm):</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>&gt; 102</td>
</tr>
<tr>
<td>Women</td>
<td>&gt; 88</td>
</tr>
<tr>
<td>Criteria 2: Triglycerides (mmol/L)</td>
<td>≥ 1.7</td>
</tr>
<tr>
<td>Criteria 3: HDL cholesterol (mmol/L)</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>&lt; 1.04</td>
</tr>
<tr>
<td>Women</td>
<td>&lt; 1.30</td>
</tr>
<tr>
<td>Criteria 4: Systolic Blood Pressure (mmHg)</td>
<td>≥ 130/ ≥ 85</td>
</tr>
<tr>
<td>Criteria 5: Fasting Blood Glucose (mmol/L)</td>
<td>≥ 6.1</td>
</tr>
</tbody>
</table>

Hcy is a homologue of the amino acid cysteine formed by altered cystathionine-beta-synthase and methylenetetrahydrofolate reductase activity and associated deficiency of folic acid, vitamin B12 and vitamin B6. Oxidation of Hcy leads to the formation of homocysteine, homocysteine-mixed disulfides, and homocysteine thiolactone, which leads to the formation of superoxide anion radicals and hydrogen peroxide (14, 15).

Elevated Hcy promotes atherosclerosis through increased oxidant stress, impaired endothelial function, and induction of thrombosis. Increases in plasma Hcy have been implicated in several disease processes including atherosclerosis affecting coronary, cerebral and peripheral arteries, chronic kidney disease (16) and Alzheimer’s disease.
Hcy-induced generation of reactive oxygen species contributes to vascular reactivity and pancreatic dysfunction leading to atherosclerosis and diabetes. The relationship between Hcy and coronary artery disease is especially pronounced in patients with diabetes.

A mechanism that has been associated with one or more of the five MetS factors, T2DM and CVD is oxidative stress and inflammation (19-21). Oxidative stress and inflammation are linked through Hcy activity (22-24). Homocysteine thiolactone reduces the generation of glycogen through oxidative stress mechanisms that have been confirmed by in vitro experiments that added GSH, a ubiquitous antioxidant, to Hcy induced oxidative stress in cell culture and reversed the reduction in insulin-stimulated glycogen synthesis (25). Hcy and oxidative stress are linked by Hcy reducing glutathione peroxidase activity, which is important for the oxidation of GSH to GSSG (26). The relationship between 8-OHdG, GSH and its oxidised form GSSG has not been extensively investigated (27-29).

Hcy is associated with increased insulin resistance, increased blood pressure and increased LDL-cholesterol, which constitute three of the five MetS factors. It therefore becomes an important question which cluster of MetS factors are present in patients with MetS and the association with Hcy and not whether MetS is present or not.

8-OHdG, a product of DNA base modification produced by the oxidation of deoxyguanosine, is considered as the most sensitive and useful marker of oxidative DNA damage (30-32). GSH is a cellular antioxidant found in red blood cells. Levels of GSH were reported to be low in patients with T2DM, possibly due to impaired activity of the γ-glutamyl-cysteine synthetase enzyme (GCS), which is involved in the biosynthesis of glutathione, a lack of cysteine availability or damage to the erythrocyte membrane, which reduces the possibility of substances required in GSH production to cross the membrane (33, 34).

Methods
A case-control study at a rural diabetes health (DiabHealth) screening clinic included 266 participants. The research was approved by the Ethics in Human Research Committee of Charles Sturt University. Participants for the study were attending the School of Community Health Diabetes Screening Clinic. All participants received an information sheet and consented to the procedure. NoMetS and MetS was defined according to the ATPIII definition. Self-reported medication use was also considered in the classification of MetS in this research. Participants were comparable for age, gender, smoking habit, diet, and physical activity. As the patient cohort were participants in a rural South West NSW and North East Victoria (Australia) diabetes screening program they were not excluded premised upon clinical and medication history.

Anthropometric data: Body mass index (BMI) was measured using standardised beam weight scales. BMI is defined as weight in kilograms per height expressed as meters squared and is independent of gender and age. Waist circumference (WC) was measured using a standard measuring tape. Measurements were taken between the top
of the hip bone and lowest rib. Blood pressure was measured using a
sphygmomanometer with appropriate cuff size after a 5-minute rest. The average of
two measurements 1 minute apart was used for systolic and diastolic blood pressure
values.

**General Biochemistry:** Blood for general biochemical analysis was collected from
volunteer clinic attendees at the screening clinic. A point of care (POCT) screening
blood glucose was performed on all patients prior to blood collection. Plasma glucose
(BGL), total cholesterol (TC), and triglycerides (TG) were determined by standard
enzymatic kits and high-density lipoprotein (HDL) was determined using an
immuno-inhibition assay. Low-density lipoprotein cholesterol (LDL-C) was calculated
according to the Friedewald formula. HbA1c, Hcy and high sensitivity CRP were
analysed by immunoassay. Assays were performed at the local NATA accredited
South West Pathology Albury Laboratory.

**Measurements of oxidative stress.** Erythrocyte MDA was measured using the
thiobarbituric acid reacting substance (35). Levels of MetHb were assessed using
spectrophotometry of hemoglobin absorption before and after cyanide addition, and
oxidative DNA damage was measured using the serum 8-OHdG ELISA Kit (Cayman
Chemical, MI, USA). The kit utilizes an anti-mouse IgG-coated plate and a tracer
consisting of an 8-OHdG-enzyme conjugate. This format has the advantage of
providing low variability and increased sensitivity compared to assays that utilize an
antigen-coated plate. The level of erythrocyte reduced GSH was determined using the
5,5’-dithiobis-2-nitrobenzoic acid (DTNB) reaction (36). Hcy was measured
quantitatively by fluorescent polarised immunoassay (FPIA) on an automated Abbott
AxSym Analyser.™ This methodology incorporates a two-step reaction with the
reduction of protein bound Hcy by dithiothreitol followed by the enzymatic
conversion of free Hcy to S-adenosyl-homocysteine in the presence of adenosine
with subsequent FPIA detection.

**Measurement of Interleukin - 6 (IL-6):** IL-6 was assayed using an Enzyme Linked
Immunosorbent Assay (ELISA) kits (Cayman Chemical, USA). The method
incorporated a double antibody sandwich methodology. Free IL-6 binds to the pre-
coated IL-6 monoclonal antibody and an acetylcholinesterase: IL-6 Fab’ conjugate is
selectively bound to a different IL-6 epitope on the molecule. The addition of AChE-
substrate (acetylcholine and 5,5’-dithio-bis-2-nitrobenzoic acid) results in colour
development measured at 412nm which is directly proportional to sample IL-6
concentration. Plates were measured using a Thermo Scientific Multiskan FC™ with
data reduction utilising SkanIt 3.1 software. Results were calculated by generating a
four parameter logistic fit curve.

**Statistical analysis.** Parametric data, premised upon kurtosis criteria, was analysed
using SPSS (Version 22) and Microsoft Excel (Office 2011, Microsoft). All values
were expressed as mean ± standard deviation (M±SD). Statistical analysis was
performed using a one-way ANOVA followed by LSD post-hoc test for between
group comparisons. Pearson correlation, taking p < 0.05 as the significance limit was
used to determine whether there was a significant correlation between Hcy and the other parameters associated with oxidative stress measured in this study.

**Results**

Traditional anthropometric and biochemical markers including WC, SBP, TC, TG, HDL, and LDL, BGLs and HbA1c were all significantly different between no MetS and MetS groups. Of the emerging biomarkers Hcy, and 8-OHdG were significantly elevated in the MetS group compared to the no MetS group (Table 2).

**Table 2. Traditional and emerging biomarkers for MetS syndrome**

<table>
<thead>
<tr>
<th></th>
<th>No MetS (n = 159)</th>
<th>MetS (n = 107)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>66±11</td>
<td>67±9</td>
<td>ns</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>92±12</td>
<td>106±11</td>
<td>0.0001</td>
</tr>
<tr>
<td>BMI (cm/kg²)</td>
<td>26±4</td>
<td>31±5</td>
<td>0.0001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>129±18</td>
<td>135±15</td>
<td>0.003</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>76.5±9</td>
<td>77.8±11</td>
<td>ns</td>
</tr>
<tr>
<td>BGL (mmol/L)</td>
<td>4.9±0.6</td>
<td>6.5±2.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.7±0.4</td>
<td>6.3±1.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>5.2±1</td>
<td>4.9±1.4</td>
<td>0.023</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.4±0.3</td>
<td>1.2±0.3</td>
<td>0.0001</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.3±0.9</td>
<td>2.8±1.2</td>
<td>0.001</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.1±0.5</td>
<td>1.9±0.9</td>
<td>0.0001</td>
</tr>
<tr>
<td>Hcy (μmol/L)</td>
<td>9.5±2</td>
<td>10.6±3</td>
<td>0.03</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>3.5±4</td>
<td>4.1±3.8</td>
<td>ns</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>31.7±27</td>
<td>41.1±45</td>
<td>ns</td>
</tr>
<tr>
<td>MDA (μmol/L)</td>
<td>13.9±7</td>
<td>16.2±8</td>
<td>ns</td>
</tr>
<tr>
<td>GSHII (mg/100mL)</td>
<td>67.6±13</td>
<td>67.8±13</td>
<td>ns</td>
</tr>
<tr>
<td>8-OHdG (pg/mL)</td>
<td>307±516</td>
<td>1130±1155</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Demographic, anthropometric and clinical results were obtained from the cohort. Table 3 shows demographic and clinical results for the 5 factors of MetS.
Table 3. Demographic and clinical data associated with metabolic syndrome factors present

<table>
<thead>
<tr>
<th></th>
<th>0 MetS</th>
<th>1 MetS</th>
<th>2 MetS</th>
<th>3 MetS</th>
<th>4 MetS</th>
<th>5 MetS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>8/20</td>
<td>26/33</td>
<td>28/44</td>
<td>30/27</td>
<td>19/18</td>
<td>7/6</td>
</tr>
<tr>
<td>(n = 28)</td>
<td>(n = 59)</td>
<td>(n = 72)</td>
<td>(n = 57)</td>
<td>(n = 37)</td>
<td>(n = 13)</td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>64.1±11</td>
<td>66.1±12</td>
<td>67.1±11</td>
<td>66.6±10</td>
<td>68.4±9</td>
<td>71.5±4</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>82.9±10</td>
<td>90.9±10</td>
<td>97.0±13</td>
<td>102.8±9</td>
<td>111.0±13</td>
<td>110.8±10</td>
</tr>
<tr>
<td>BMI (cm²/kg²)</td>
<td>24±2</td>
<td>26.1±4</td>
<td>28.7±4</td>
<td>30.1±5</td>
<td>32.4±5</td>
<td>31.3±4</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>114±12</td>
<td>128±13</td>
<td>135.1±20</td>
<td>134.8±16</td>
<td>135.8±14</td>
<td>137.5±14</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>70.6±7</td>
<td>75.9±6</td>
<td>79.6±11</td>
<td>78.8±11</td>
<td>77.1±9</td>
<td>74±10</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.6±0.3</td>
<td>5.7±0.4</td>
<td>5.7±0.4</td>
<td>6±0.6</td>
<td>6.5±2</td>
<td>7±2</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>5.3±0.9</td>
<td>5.4±1</td>
<td>5.1±1</td>
<td>5.1±1</td>
<td>4.7±1</td>
<td>4.4±1</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.6±0.2</td>
<td>1.4±0.3</td>
<td>1.4±0.3</td>
<td>1.3±0.3</td>
<td>1.1±0.2</td>
<td>1±0.1</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>3.4±0.8</td>
<td>3.5±0.9</td>
<td>3.2±0.9</td>
<td>3.1±1</td>
<td>2.8±1</td>
<td>1.9±1</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>0.8±0.3</td>
<td>1±0.4</td>
<td>1.2±0.5</td>
<td>1.6±0.8</td>
<td>2±0.8</td>
<td>3.1±0.9</td>
</tr>
<tr>
<td>BGL-lowering (%)</td>
<td>0</td>
<td>1.6</td>
<td>5.5</td>
<td>22.8</td>
<td>40.5</td>
<td>53.8</td>
</tr>
<tr>
<td>Chol-lowering (%)</td>
<td>0</td>
<td>8.4</td>
<td>22.2</td>
<td>31.5</td>
<td>62.1</td>
<td>61.5</td>
</tr>
<tr>
<td>BP-lowering (%)</td>
<td>0</td>
<td>22</td>
<td>45.8</td>
<td>61.4</td>
<td>83.7</td>
<td>76.9</td>
</tr>
</tbody>
</table>

All clinical markers increased with respect to the number of MetS factors present except LDL-cholesterol, most likely due to the increase in cholesterol lowering medication use. HDL-cholesterol decreased in line with worsening pathology with increasing MetS factors present. Age was not significantly different between groups. WC was significantly different between all MetS factor groups (p<0.01), except for 4 MetS versus 5 MetS. In addition the group with no MetS factors present was significantly different from all groups with one to five MetS factors present (P=0.0001). DBP for the no MetS factors group was significantly different from the groups with one to four MetS factors present (p<0.05). Fasting BGL rose steadily and showed significant differences between no MetS factors and three to five MetS factors present (p=0.0001). Significant increases were also observed between 1 MetS and 3, 4 and 5 MetS factors present (p=0.0001); 2 MetS versus 3, 4 and 5 MetS (p=0.0001); 3 MetS and 5 MetS (p=0.0001) and between 4 MetS and 5 MetS (p=0.0001). Of the cholesterol markers, the triglycerides and HDL-cholesterol were most affected by addition of a metabolic risk factor, with TG’s rising significantly between groups, whilst HDL decreased (p<0.02). LDL-cholesterol also increase between 0 MetS versus 4 and 5 MetS (p<0.03); Between 1 MetS versus 3, 4 and 5 MetS (p<0.03) and between 2, 3 and 4 MetS versus 5 MetS (p<0.02).

Levels of inflammatory and oxidative stress markers as a function of the number of MetS factors present are shown in Table 4.
Table 4. Inflammatory and Oxidative stress markers in metabolic syndrome

<table>
<thead>
<tr>
<th></th>
<th>0 MetS</th>
<th>1 MetS</th>
<th>2 MetS</th>
<th>3 MetS</th>
<th>4 MetS</th>
<th>5 MetS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hcy (μmol/L)</td>
<td>8.6±3</td>
<td>9.5±3</td>
<td>9.7±3</td>
<td>10.9±3</td>
<td>10.6±3</td>
<td>9.4±3</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>2.1±3</td>
<td>4.1±6</td>
<td>3.7±2</td>
<td>4.7±5</td>
<td>3.1±2</td>
<td>3.8±3</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>29±20</td>
<td>38±30</td>
<td>24.4±29</td>
<td>129.4±261</td>
<td>46.3±29</td>
<td>30±27</td>
</tr>
<tr>
<td>MDA (μmol/L)</td>
<td>14.7±6</td>
<td>14.2±8</td>
<td>12.8±6</td>
<td>15.2±9</td>
<td>17.8±9</td>
<td>16.7±8</td>
</tr>
<tr>
<td>GSH (mg/100mL)</td>
<td>67±11</td>
<td>67.1±16</td>
<td>68.7±11</td>
<td>69.2±12</td>
<td>65±12</td>
<td>70.1±15</td>
</tr>
<tr>
<td>8-OHdG (pg/ml)</td>
<td>212±163</td>
<td>199±140</td>
<td>459±763</td>
<td>953±1110</td>
<td>1063±1076</td>
<td>1711±1403</td>
</tr>
</tbody>
</table>

Inflammation and oxidative stress increases with the number of MetS factors present in the cohort. The most dramatic increase was seen in 8-OHdG, which nearly doubled between 2 MetS and 3 MetS factors present (p<0.001). CRP rose significantly between 0 MetS versus 1 MetS and 3 MetS (p<0.05). MDA is a general oxidative stress marker and was significantly different between 2 MetS and 4 MetS only (p=0.01). In the current study, GSH showed a steady rise initially with increasing number of MetS factors present but no significant changes were noted.

Hcy levels steadily increased up to any 3 MetS factors present and then declined slightly (Figure 1). Significant differences for Hcy were found between no MetS factors present 3 (p=0.001) and 4 MetS factors (p=0.007) present as well as between 1MetS and 3MetS (p=0.013) and 2MetS versus 3MetS (p=0.034). A decrease in Hcy levels were observed between 4 and 5 MetS factors present but this decline did not reach significance.

Fig 1

Figure 1 provides a comparison of the levels of the biomarkers Hcy, 8-OHdG and GSH relevant to the number of MetS factors present.
Discussion
The NCEP ATP III definition is a useful tool for the classification of MetS patients as it can be easily applied in a clinical setting by physicians scoring on the 5 criteria using easily measured endpoints and without the use of more complex algorithms or computations (3).

The categorisation of MetS using, for example, the World Health Organization (WHO), the European Group for the Study of Insulin Resistance (EGIR) or the International Diabetes Federation (IDF) classifications of MetS requires further consideration of parameters such as insulin resistance, hyperinsulinemia and microalbuminuria. As the aim of this study was to investigate MetS in a screening clinic, with patients not excluded on the basis of treatment, clinical status, or the measurement of insulin resistance the ATPIII classification was deemed most appropriate for our rural screening clinic study.

An important caveat of the current study is that in addition to the ATPIII definition of our MetS cohort glucose-lowering, blood pressure-lowering and cholesterol-lowering medication use was used as further criterion for inclusion in the hyperglycemia, increased blood pressure and increased cholesterol factors of MetS. Our reasoning for this was that we concentrated on a population sample to determine changes in Hcy and whether these were significant and clinically usable as indicators of CVD and diabetes risk. Previous work suggests that 8-OHdG and GSH may be a useful clinical indicators. Endothelial function and insulin resistance was improved in patients with metabolic syndrome after folate and Vitamin B12 therapy (37). Homocysteine thiolactone also decreased in culture cells when GSH was added to the medium and improved insulin signalling (25).

It is widely acknowledged that both age and gender impact on the prevalence and prognosis of MetS (38), however current protocols do not consider them or other parameters such as LDL and emerging metabolic factors such as oxidative stress, inflammatory and coagulation markers (10, 39). Endothelial dysfunction is a hallmark of CVD and T2DM suggesting that markers indicating endothelial dysfunction may be clinically relevant in assessing metabolic syndrome and risk of CVD and T2DM. Hcy is one such marker that is found in increased concentration in blood due to genetic or pathophysiological processes (14). By-products of Hcy degradation then play a role in oxidative stress (19). 8-OHdG and GSH have been shown to be associated with increased fasting BG levels, one of the factors in MetS (10, 40, 41). Increased Hcy levels appear to be concomitant with MetS due to a relationship with diabetes progression (42, 43), however as a standalone test for MetS progression, Hcy itself may be predictive of MetS development per se as it is associated with hypertension and therefore higher levels are suggestive of its concomitance with MetS (42).

Hcy may be a useful marker together with 8-OHdG in assessing the extent of metabolic syndrome and the impact of medication especially in a clinical cohort, where clinical parameters need to be highly correlated with disease. Thus, for example, elevated results of systolic blood pressure are indicative of hypertension and associated pathophysiology regardless of whether other chronic disease is present or whether patients are on antihypertension medication. This principle was borne out in our findings that 8-OHdG levels did show increased significance despite medication

323
usage, indicating that markers such as 8-OHdG warrant further investigation into heterogeneous populations when investigating MetS, T2DM and CVD progression. Our results also confirm previous findings that Hcy is not correlated with 8-OHdG (44) nor with GSH. However GSH increased steadily up to four MetS factors when it decreased dramatically and then increased when five MetS factors were present. The decrease may be an artefact but may also be a result of downregulation by Hcy, which steadily increased with addition of MetS factors. GSH, which is oxidised to GSSG in the presence of hyperglycaemia may also have contributed to the drop seen in GSH levels in addition to the antioxidant effects of medication use. We propose that GSH displays a cyclic change with disease progression, first increasing as oxidative stress increases but decreasing with increased oxidative stress prior to de novo synthesis, which leads to a secondary increase in GSH (39, 45, 46). This secondary increase in GSH is further enhanced due to the decrease in Hcy as seen in our study, which may be due to the increase in medication (Table 2) (47).

Results were not adjusted for age, gender or creatinine as reported previously in a study comparing Hcy levels in people with MetS and vascular disease as our study concentrated on determining whether Hcy is a suitable marker for MetS in a population diabetes health (DiabHealth) screening initiative. We found significant results for Hcy associated with either MetS (≥3 factors present) versus noMetS (<3 factors present) in the cohort as well as a relationship between Hcy levels and an increase in the number of MetS factors included in the analysis, which was also reported previously (48). However appropriate cut-off can be determined in a larger study considering age and gender.

Whether Hcy is a causative factor in disease development or simply a biomarker is controversial with possibly links in diabetic nephropathy (49, 50), muscle malfunction (51), Alzheimer’s disease (52) and other comorbidities associated with MetS development (42). Our study indicates that the significant results associated with Hcy and the clustering of MetS factors may be the consequence of complex pathophysiological metabolic interactions with 8-OHdG and glutathione, suggesting that pathological vascular oxidative processes become more pronounced with the increase in MetS factors. This is associated with the presence of elevated levels of Hcy in atherosclerosis and other hypercoagulability states (53).

Conclusion

This study has demonstrated the utility of emerging oxidative stress and inflammatory markers in assisting clinicians in identifying the development of T2DM and CVD as a consequence of MetS progression. The aim of this study was to observe if community screening of patients attending a rural diabetic clinic who were not excluded on the basis of clinical or medication history provided results comparable to homogenous controlled population studies. Further research is required on these emerging markers but early indications are there may be useful as part of a screening tool in disease progression for at risk patients with evidence of MetS.

In recognition that the existence of MetS as an entity is still controversial, and that differing definitions have and do provide an increasing insight into this syndrome (54), our selection of the ATPIII guidelines in conjunction with emerging biomarkers may provide a useful clinical approach into screening a population who are potentially more at risk for the development of T2DM and CVD.
Acknowledgements
Bev de Jong is gratefully acknowledged for her contribution collecting the data in the diabetes health clinic and Simon McDonald from the Spatial Analysis Unit at CSU for providing statistical support. Roche Australia provided the glucose measuring sticks and glucometers.

Conflict of Interest
The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

References

48. Hajer GR, van der Graaf Y, Olijhoek JK, Verhaar MC, Visseren FL. Levels of homocysteine are increased in metabolic syndrome patients but are not associated with an increased cardiovascular risk, in contrast to patients without the metabolic syndrome. Heart (British Cardiac Society). 2007;93(2):216-20.
Appendix J: Association of Inflammation and Possible Mild Cognitive Decline. Measured by the Stroop Cognitive Function Test

Association of Inflammation and Possible Mild Cognitive Decline Measured by the Stroop Cognitive Function Test

Fabrégué F1,2, Butkowski E1, Volgt A1,2, Mouquet G1,2, de Jong B3, Stachowiak FJ1 and Jellinek HF1,2
1Faculty of Sciences, University of Poitiers, Poitiers, France
2School of Community Health, Charles Sturt University, Albury, Australia
3Biomolecular Chemistry and Biochemistry, Free University of Berlin, Germany
4School of Advanced Medicine, Macquarie University, Sydney, Australia

Abstract
Alzheimer’s disease and dementia have been shown to be associated with various inflammatory markers. However, the association between mild cognitive decline (MCI) and inflammation is not conclusive. Determining MCI requires a battery of tests of which the Stroop battery is one that is often used to assess cognitive function. The current study investigated the level of inflammation and the correlation to the RIT and NIT assessment of MCI in a rural cohort attending a health screening clinic.

Ninety-six participants free of diagnosed MCI undertook the Stroop testing battery and were divided into a short reaction time (SRT) and a long reaction time (LRT) group based on the RIT and NIT results. Serum Interleukin (IL-10, IL-6, IL-1β), Insulin-like Growth Factor-1 (IGF-1), C-Reactive Protein (CRP) and the Monocyte Chemotactic Protein-1 (MCP-1) levels were measured using commercial ELISA kits.

CRP was significantly higher in the LRT group compared to the SRT group (p = 0.023). IL-1β was associated with NIT (p = 0.036). MCP-1 was significant only for NIT (p=0.01). The IL-10 (p=0.01) was significantly lower (p=0.0098) and the IL-6/L-10 ratio significantly higher in the LRT group regardless whether RIT or NIT was used (p=0.03).

The study extends previous work indicating an association between cognitive function measured by the Stroop battery and inflammation. IL-10 and the IL-6/L-10 ratio are the most appropriate markers to use to assess the level of inflammation associated with reaction time and hence cognitive function.

Keywords: Inflammation; Mild cognitive impairment; Stroop test; Reading Interference test; Naming Interference test

Abbreviations: IL: Interleukin; IGF-1: Insulin-like Growth Factor-1; CRP: C-Reactive Protein; MCP-1: Monocyte Chemotactic Protein-1; NIT: Naming Interference Test; RIT: Reading Interference Test

Introduction
Many factors such as cerebrovascular accidents, hypothyroidism, heart failure, vitamin deficiencies, hepatic impairment, depression, drug and alcohol abuse, toxins, infections, metabolic and structural causes influence cognitive function that can lead to mild cognitive impairment, dementia and Alzheimer’s disease. Furthermore MCI is also linked to obesity, vascular disease, dyslipidemia, inflammation and oxidative stress [1-4]. Mild cognitive impairment (MCI) is a common age related phenomenon, which may be a pre-clinical stage of dementia in general and Alzheimer’s Disease (AD) specifically. Episodic memory is most often affected by cognitive impairment, which can lead to AD dementia [5]. How MCI is identified and whether it is affected by dyslipidemia, inflammation and oxidative stress is important in clinical practice as it can determine treatment priorities.

Oxidative stress and inflammation associated with vascular disease have been proposed to be early markers of cognitive decline and one study suggested that the level of oxidative markers is directly related to the severity of cognitive impairment [6]. Previous research has also shown a possible link between oxidative stress and cytokine production [7]. Expression of inflammatory cytokines in neuronal cells suggests that inflammation and the production of ROS are closely related in the progression of cognitive decline [8]. Cholesterol has also been implicated with a causative role in MCI [9]. However controversy still exists whether inflammation and MCI are related [10].

Cognitive tests are commonly used to determine the presence of MCI. Scores on cognitive tests for individuals with MCI are typically 1 to 1.5 standard deviations below the mean for their age and education matched peers on culturally appropriate normative data [5]. Examples of such test batteries include the Differential Aptitude Test Battery, Cambridge Neuropsychological Test Automated Battery (CANTAB®) [11] and the Vienna Cognitive Test Battery [12].

The Vienna Cognitive Test Battery includes a number of tests for attention, memory and visuospatial skills of which the color-word interference tendency, (Stroop) test has been extensively investigated and often used as part of a test battery to diagnose MCI and vascular disease [13]. The principle of the Stroop test [14] is that it is harder for an individual, to name the color of the writing if the word is written in a different color than if naming the color of the word written in the same color. Test results are interpreted by the median reaction time (seconds) and/or the number of incorrect responses made in relation to congruent and incongruent words. The interference between congruent and incongruent words can then be a predictor of MCI as interference deficits may be present before cognitive decline symptoms reach

*Corresponding author: Jellinek HF, School of Community Health, Charles Sturt University, Albury, Australia, Tel: 61427681754; E-mail: hjellinek@csu.edu.au

Received May 11, 2016; Accepted May 20, 2016; Published May 27, 2016


Copyright: © 2016 Fabrégué F, et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

J Alzheimers Dis Parkinsonism
ISSN:2161-0440 JAD® an open access journal

Volume 6 Issue 3 - 1000237
clinical levels [15]. The results of the Stroop test can then be applied to determine whether certain inflammatory markers are raised or deficient in MCI.

Cytokines are inflammation biomarkers, which play a role in enhancing or preventing disease progression. An imbalance of pro- and anti-inflammatory cytokines, which may be caused by dyslipidemia and leading to inflammatory cascades is related to changes in the levels of interleukin-6 (IL-6) and increases in other cytokines such as interleukin-1β (IL-1β) production [16]. The pro-inflammatory IL-1β is a crucial factor contributing to MCI and is a possible tool to predict neurodegenerative diseases [17].

Much of the current published research on MCI investigated only one specific biomarker and reported associations with either the Stroop RIT or NIT scores but did not apply both Stroop tests. C-reactive protein (CRP) [18], Interleukin-12 (IL-12) [19], Interleukin-1 (IL-1) [20], tumor necrosis factor-α (TNF-α) [21] and monocyte chemotactic protein-1 (MCP-1) [22] have been shown to significantly increase or decrease in MCI. The link between pro-inflammatory and anti-inflammatory biomarkers and cognitive decline has only been shown in one study of patients with early or late onset Alzheimer’s disease (LOAD) [23], reporting a significant higher serum IL-6 level in the LOAD group and a significant correlation with high serum IL-10 levels. In addition results of previous research investigating the relationship between MCI and inflammation are not conclusive and may be nonspecific [10,24]. The role of anti-inflammatory biomarkers and their interaction with pro-inflammatory cytokines has not been investigated in the context of Stroop results [25,24]. The current study therefore aimed at investigating the association of pro- and anti-inflammatory biomarkers using both RIT and NIT to determine whether an association exists between various inflammatory markers and the Stroop RIT and NIT score.

Methods

The study was approved by the University Ethics in the Human Research Committee (Ethics approval number: 2006/042). Informed consent was obtained from each participant after providing a comprehensive overview of the project and methods followed by answering any questions participants may have had.

Study population

For the present study, 132 participants were recruited attending a rural health screening clinic for cognitive function testing using the Stroop test battery and analysis of blood biomarkers. We divided the results into a low reaction time (LRT) and high reaction time (hRIT) group based on the RIT or NIT results. Twenty-nine patients were excluded because blood samples were not available, while seven others did not complete the cognitive function tests. The final number of participants for the study was 96. The cut-off values for hRIT using RIT or NIT were determined from 25 patients attending the health screening clinic who reported no hypertension (HT), cardiovascular disease (CVD), diabetes, psychiatric disease or cognitive difficulty and presented the baseline values. The cut-off values for the reading interference test (RIT) and naming interference test (NIT) were then determined by setting the T-score value of the Stroop test results for RIT and NIT equal to 60, which represents one standard deviation from the normalized mean of the baseline group. This provided a cut-off for RIT of >240 milliseconds (ms) and for NIT of >680 ms. Scores below the cut-off were deemed as lowRIT and represent the 85th centile for the current cohort.

Comorbidities presenting in patients such as diabetes were defined as fasting plasma glucose levels (FPG) ≥ 126 mg/dL (7 mmol/L) [27]. Hypertension was defined as a systolic blood pressure (SBP) ≥ 140 mmHg or self-reported hypertension with or without medication use. CVD was identified by 12-lead ECG, self-reported clinical history and use of medication.

Chemicals

The blood samples were analyzed using ELISA kits (Eli Titan, Melbourne, Australia) [19,20]. Interleukin-12 (IL-12) (human) [21], Interleukin-1 (IL-1) (human) [22], tumor necrosis factor-α (TNF-α) [23] and monocyte chemotactic protein-1 (MCP-1) [24] have been shown to significantly increase or decrease in MCI. The link between pro-inflammatory and anti-inflammatory biomarkers and cognitive decline has only been shown in one study of patients with early or late onset Alzheimer’s disease (LOAD) [23], reporting a significant higher serum IL-6 level in the LOAD group and a significant correlation with high serum IL-10 levels. In addition results of previous research investigating the relationship between MCI and inflammation are not conclusive and may be nonspecific [10,24]. The role of anti-inflammatory biomarkers and their interaction with pro-inflammatory cytokines has not been investigated in the context of Stroop results [25,24]. The current study therefore aimed at investigating the association of pro- and anti-inflammatory biomarkers using both RIT and NIT to determine whether an association exists between various inflammatory markers and the Stroop RIT and NIT score.
Results

Anthropometric data for the 96 adults who completed the cognitive function tests and for whom blood samples were available is shown in Table 1. All participants were older than 40 years. Sixty-two per cent of the study participants in the low reaction time Stroop group were women versus 35.7% in the high reaction time Stroop group. The mean age of the low reaction time Stroop group was 67 years and not significantly different to the high reaction time Stroop group. Only CVD and HDL-Cholesterol were significantly different between the low and high reaction time Stroop group (p<0.05).

For the RIT cut-off was 240 ms and the cut-off for NIT was 668 ms. Reaction times were determined based on the RIT and NIT Stroop results and presented separately.

Mean cytokines levels were determined with respect to the RIT and NIT test results. For RIT, serum CRP levels were significantly higher in the high reaction time Stroop group compared with the low reaction time Stroop group (increase of 50%, p=0.022) (Figure 1) and IL-10 was significantly lower with a decrease of 16.7% (p=0.0096).

Values shown in the bar plots are the normalized means ± SD of all inflammatory biomarkers. The white bar plots represents the participants, who have a RIT value below the cut-off (<240 ms). The grey bar plots represents the participants, who have a RIT value above the cut-off (>240 ms).

MCP-1 was significantly lower in the high reaction time Stroop group when the NIT classification was applied, (7.8%, p=0.003). The high reaction time Stroop group also demonstrated a significant increase in IL-1β levels of 49% (p<0.039) but IL-1β levels were not significantly increased on the basis of RIT increase of 36% compared Figures 1 and 2).

Only IL-10 levels decreased significantly regardless of the Stroop test (RIT: p=0.0096 and NIT: p=0.003).

The interactions between pro- and anti-inflammatory markers are an important component of disease progression. To obtain an understanding of this balance we used the ratio between IL-6, a pro-inflammatory cytokine and IL-10, an anti-inflammatory cytokine.

<table>
<thead>
<tr>
<th>Low Stroop</th>
<th>High Stroop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (women) 62.2</td>
<td>63.7</td>
</tr>
<tr>
<td>Age (years) 67.0 ± 9.5</td>
<td>68.7 ± 10.7</td>
</tr>
<tr>
<td>Glucose (mmol/L) 6.9 ± 2.8</td>
<td>6.8 ± 1.8</td>
</tr>
<tr>
<td>BMI (kg/m²) 28.6 ± 5.8</td>
<td>30.6 ± 5.4</td>
</tr>
<tr>
<td>CVD (%) 27.2 ± 14.5</td>
<td>102.9 ± 9.8</td>
</tr>
<tr>
<td>CVD (%) 27.2 ± 14.5</td>
<td>102.9 ± 9.8</td>
</tr>
<tr>
<td>Type 2 Diabetes (%) 59.7</td>
<td>50.0</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L) 7.0 ± 1.1</td>
<td>5.5 ± 1.3</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L) 1.9 ± 0.8</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg) 131.3 ± 17.9</td>
<td>130.9 ± 13.4</td>
</tr>
</tbody>
</table>

Figure 3 indicates an increase in the IL-6/IL-10 ratio when applying RIT of 8% (p=0.016) and an increase of 40% for NIT (p=0.019).

Values shown in bar plots are the normalised means ± SD of the ratio IL-6/IL-10.

Discussion

Cognitive decline due to vascular impairment without dementia as a disease entity has gained increased recognition in clinical practice. MCI can be diagnosed by a battery of tests including the Stroop test. Our results indicate that whether a difference in inflammation markers is observed between the low reaction time compared to the high reaction time Stroop group based on the Stroop test is observed depends on whether the RIT or NIT is used.

An elevated Stroop reaction time score and therefore a possible risk of developing MCI were linked with a higher mean CRP level and a lower IL-10 level when the RIT score was used. Higher reaction time scores with the NIT test were significantly associated with lower MCP.
IGF-1 was shown to be increased in association with the NIT in a cohort of 61 MCI patients, suggesting that IGF-1 is part of a complex response pattern [35]. We found no significant increase in IGF-1 in the hIRT group but according to the literature, IGF-1 can also be a protecting factor against MCI suggesting that in our study IGF-1 may be on the rise or already decreased due to its protective function in the high reaction time group [36]. A future study investigating progression of MCI may be able to clarify whether IGF-1 increases as MCI progresses.

IL-6 was not significantly increased in our hIRT group regardless of whether MCI was defined on the basis of a high RIT or NIT score. However, IL-6 was close to being significantly increased when hIRT was determined by NIT (p=0.052), supporting an increased IL-6 level reported previously in MCI patients [24]. The elevated IL-6 and IL-10 found in the hIRT group based on the NIT results. The difference in whether IL-1β was significantly increased in the hIRT group depended on which of the two Stroop tests was used (RIT versus NIT) for defining the high reaction time group. This highlights the need to be aware that cognitive function test results may reflect different neurological functions as suggested by the current results following the reading and naming interference tests.

Mean levels of the anti-inflammatory marker MCP-1 were significantly lower for the NIT. Our results in this case are in agreement with some previous studies that have shown MCP-1 to be increased in MCI patients [22]. However, Gaitaníberti et al. [22] demonstrated that MCP-1 elevation is a very early event in AD pathogenesis and decreases with AD progression. The findings of Gaitaníberti et al. [22] suggest that NIT may be a sensitive test for early AD. However, the current study did not include MCI or AD patients but investigated the association between inflammation and low or high reaction time determination with the Stroop battery. Therefore, the significantly lower MCP-1 level observed in the high reaction time group suggests a possibly different pathophysiology compared to that of developing AD.

We also studied the association of the anti-inflammatory IL-10 and reaction times on the Stroop test. IL-10 acts to diminish pro-inflammatory cytokines [32]. What the role of IL-10 is in MCI pathology is not clear. Previous results have shown an increase in a decrease in IL-10 in association with Parkinson’s disease and multiple sclerosis that may have an MCI component [33]. Experiments with lipopolysaccharide (LPS) activated macrophages increased the level of IL-10 in AD patients [34]. In our study, participants with known Parkinson’s disease and multiple sclerosis were excluded and thus the significantly lower IL-10 levels may be due to IL-10 acting as an anti-inflammatory cytokine in the hIRT group and is decreased due IL-10 binding to certain transcription factors that decrease inflammatory activity or the increased reaction time observed in this group may be linked to IL-10 hypo-responsiveness.
Appendix K: Acute-Phase Inflammatory Response to Single-Bout HIIT and Endurance Training: A Comparative Study

Research Article

Acute-Phase Inflammatory Response to Single-Bout HIIT and Endurance Training: A Comparative Study

Felix Kaspar,1,2 Herbert F. Jelinek,2,3 Steven Perkins,2 Hayder A. Al-Aubaidy,4 Bev de Jong,2 and Eugene Butkowski2

1Department of Biotechnology, Institute for Biochemistry and Biotechnology, Technical University Braunschweig, 38126 Braunschweig, Germany
2School of Community Health, Charles Sturt University, Albury, NSW 2640, Australia
3Australian School of Advanced Medicine, Masquerie University, Sydney, NSW 2019, Australia
4School of Medicine, University of Tasmania, Hobart, TAS 7001, Australia

Correspondence should be addressed to Herbert F. Jelinek; hjelinek@csu.edu.au

Received 24 February 2016; Revised 1 April 2016; Accepted 3 April 2016

Academic Editor: Pulvio D’Acquisto

Copyright © 2016 Felix Kaspar et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Objective. This study compared acute and late effect of single-bout endurance training (ET) and high-intensity interval training (HIIT) on the plasma levels of four inflammatory cytokines and C-reactive protein and insulin-like growth factor 1. Design. Cohort study with repeated-measures design. Methods. Seven healthy untrained volunteers completed a single bout of ET and HIIT on a cycle ergometer. ET and HIIT sessions were held in random order and at least 7 days apart. Blood was drawn before the intercessions and 30 min and 2 days after the training sessions. Plasma samples were analyzed with ELISA for the interleukins (IL)-1β, IL-6, and IL-10, monocyte chemoattractant protein-1 (MCP-1), insulin growth factor 1 (IGF-1), and C-reactive protein (CRP). Statistical analysis was with Wilcoxon signed-rank tests. Results. ET led to both a significant acute and long-term inflammatory response with a significant decrease at 30 minutes after exercise in the IL-6/IL-10 ratio (−30%; p = 0.047) and a decrease of MCP-1 (−17%; p = 0.03). Conclusion. This study demonstrates that ET affects the inflammatory response more adversely at 30 minutes after exercise compared to HIIT. However, this is compensated by a significant decrease in MCP-1 at two days associated with a reduced risk of atherosclerosis.

1. Introduction

Exercise and general physical activity are commonly associated with a healthy lifestyle and longevity as well as overall low levels of proinflammatory and high levels of anti-inflammatory cytokines [1].

Cytokines of the interleukin group, including interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin 10 (IL-10), are key agents of the immune system and are involved in the systemic response to local inflammation [1]. Single exercise sessions of varying intensity and duration have been shown to induce systemic inflammatory responses similar to those associated with injury [2, 3].

The acute-phase response (APR) is the body's immediate response to inflammatory stimuli such as strenuous exercise and includes a complex mediator cascade aimed towards minimizing expansion of tissue damage and enabling recovery from proinflammatory processes. Exercise activates monocytes and macrophages with subsequent upregulation and expression of proinflammatory cytokines such as IL-1β, IL-6, and monocyte chemoattractant protein-1 (MCP-1) [4, 5]. MCP-1 levels, as well as those of the anti-inflammatory IL-10, have been shown to increase during and following exercise in order to contain inflammation and restore normal physiological function of the affected tissue [6–8]. IL-1β is also effective in the APR, proinflammatory cell activation, and stimulation of APR gene expression [4]. Despite its principal role in the APR, findings of exercise-induced IL-1β increases have been inconsistent [5]. Since IL-1β increases not only its own production but that of IL-6 and C-reactive protein (CRP) as well, elevated levels of this cytokine may have adverse effects in the context of inflammation containment [5, 9].
Physiological levels of C-reactive protein (CRP) are generally very low in healthy resting individuals but can increase up to 10,000 times during the APR [10]. Previous studies on the relationship between single bouts of exercise and the CRP response have revealed conflicting data as a number of studies demonstrated postexercise CRP level reductions, whereas others failed to do so [11, 12]. Further, the physiological levels of anabolic hormones such as insulin growth factor 1 (IGF-1), a pivotal mediator of muscle hypertrophy [13], appear not to change following a single bout of endurance or resistance exercise [2, 14].

High-intensity interval training (HIIT) is a type of workout regimen that has recently become popular due to its time effectiveness compared to traditional more time-consuming forms of endurance training (ET). HIIT yields similar training results such as an increase in mitochondrial enzyme activity, muscle oxidative capacity, and muscle glycogen content to conventional endurance training approaches by compensating the reduction in training volume with an increase in exercise intensity [8, 15–17].

Although the effects of single bout ET on inflammation have been extensively reported, studies on inflammatory responses to HIIT either investigated the systemic responses to repeated sessions [6] or examined HIIT in isolation [8] or in combination with other interfering stress factors including additional training volume and cumulative fatigue [18]. The overall consensus of these studies was that low-volume HIIT appears to lead to a similar inflammatory response to ET and supplies a sufficient training stimulus to generate significant physiological adaptations comparable to those achievable with endurance training in nonoverreaching individuals.

To gain a better understanding of the acute and medium-term inflammatory responses to single exercise sessions and whether these may be related to the type of exercise, we investigated the effect of single-bout HIIT and ET on five inflammatory cytokines and IGF-1 in a group of young untrained adults. We hypothesized that HIIT may cause a different inflammatory response compared to ET due to its larger impact on heart rate and highly exhaustive character.

2. Methods

All participants were informed of the testing and training procedures and the potential risks involved. Participation was fully voluntary and participants could withdraw from the study at any time. Written informed consent was obtained prior to study commencement. All participants were instructed to abstain from exercise for 48 h preceding and following the exercise intervention. Consumption of alcohol and caffeine was prohibited for the duration of the study. This study was approved by the Human Research Ethics Committee (Charles Sturt University, Albury, Australia), protocol number 2014/161.

Nine healthy university students (Table 1) were recruited from student lectures two weeks prior to testing. Inclusion criteria were age 18–30 years, body mass index 18–30 kg·m⁻², blood pressure 90/60–140/90 mmHg, not taking any medications or supplements including anti-inflammatory drugs, not currently diagnosed with a chronic health condition, nonsmoking, and being untrained (not training more than once per week at high or moderate intensity). Prior to the exercise bouts, participants were screened to ensure that they fit the inclusion criteria and that it was safe for them to undertake a high-intensity exercise program in accordance with the Exercise and Sports Science Australia Adult Pre-Exercise Screening Tool (2011) stages 1 and 2.

After completing the preliminary screening, participants were familiarized with the equipment used for the exercise protocols. Participants were randomly assigned to perform either the ET or the HIIT protocol first at least three days following the familiarization. The ET and HIIT sessions were performed at least seven days apart and in the morning (between 8 a.m. and 12 noon). This 7-day washout period between the sessions was applied to ensure cytokine levels had returned to baseline before beginning the second intervention based on results by Ostrowski et al. [3], who described 5 days to be sufficient for cytokine levels to normalize.

The ET and HIIT sessions consisted of single supervised morning exercise sessions performed on an air-braked cycle ergometer (Wattbike Ltd., Nottingham, UK).

<table>
<thead>
<tr>
<th>Table 1: Subject characteristics.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
</tr>
<tr>
<td>Gender, M/F</td>
</tr>
<tr>
<td>Weight (kg)</td>
</tr>
<tr>
<td>Height (cm)</td>
</tr>
<tr>
<td>Body mass index (kg·m⁻²)</td>
</tr>
<tr>
<td>Blood glucose level (mmol·L⁻¹)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
</tr>
<tr>
<td>HDL (mmol·L⁻¹)</td>
</tr>
<tr>
<td>LDL (mmol·L⁻¹)</td>
</tr>
<tr>
<td>Total cholesterol (mmol·L⁻¹)</td>
</tr>
<tr>
<td>Triglycerides (mmol·L⁻¹)</td>
</tr>
</tbody>
</table>

Data is presented as mean ± SD. SBP: systolic blood pressure. DBP: diastolic blood pressure. HDL: high density lipoprotein. LDL: low density lipoprotein.

The HIIT session involved 2 min warm-up at <50 watts followed by 6 sets of 30 s of all-out supramaximal intensity cycling at the participant’s respective self-selected gearing. Between sets participants completed a 4 min recovery period in which they either rested or cycled below 30 watts.

The ET session consisted of 45 min of ergometer cycling at a moderate intensity, which was calculated at 62.5% of maximal heart rate. Maximum heart rate was calculated according to the formula suggested by Tanaka et al. (208 – 0.7 age) [19]. Heart rate was measured by a heart rate monitor (RS800CX; Polar Electro Ltd.) with chest strap. Participants were able to monitor their current heart rate and were asked to maintain constant exercise intensity at their calculated heart rate.
Baseline blood samples were taken after 5 min of rest in a seated position on the day of the first intervention. Postexercise blood samples were taken 30 min and 2 days after completion of the ET and HIIT protocol. At each time point, 20 mL of blood was drawn from the antecubital vein in two 10 mL EDTA-tubes, centrifuged for 15 min at 800 g and 4°C. The plasma was transferred into 2 mL Eppendorf tubes (Eppendorf AG, Germany) and stored at −80°C until analysis (within 4 months).

Each sample was analyzed in duplicate using commercially available sandwich ELISA kits (http://www.elsakits.com) in compliance with the supplier’s instructions. According to the information provided by the manufacturer, the lower limit of quantification of the assays was less than 1 pg/mL⁻¹ for IL-1β, less than 5 pg/mL⁻¹ for IL-6, IL-10, and IGF-1, and less than 10 pg/mL⁻¹ for MCP-1. The intra- and interassay coefficient of variance was <10% for all assays. The Lot numbers were IL-1β: #P-150702; IL-6: #P-150432; IL-10: #P-150409; IGF-1: #P-150512; MCP-1: #P-150417. The optical density at 450 nm was measured with a Multiskan FC Microplate Photometer (Thermo Fisher Scientific Inc., Waltham, MA).

Serum CRP levels and cholesterol profile were provided by Doerrich Pathology Laboratory, Albury, NSW. Blood glucose levels were measured by BGL meters (Hoffmann-La Roche, Basel, Germany) from finger blood samples.

Data were analyzed with Microsoft Excel (Office 2010, Microsoft), SPSS (Version 22, IBM Inc.), and S-Plus 8 (TIBCO, Seattle, Washington). As participant numbers were below 10, the Wilcoxon signed-rank tests were applied to determine whether there were significant changes in the biomarker levels for post-END and post-HIIT and between ET and HIIT. Correlation analyses were performed with Origin (OriginLab, Origin ProLab 9) and SPSS (SPSS v20, IBM). A p value < 0.05 was considered as significant. To determine the association between CRP, IL-6, and IL-1β, the synergistic model recommended by Ganter et al. was used [9].

Descriptive statistics for continuous variables were calculated in Excel and are expressed as mean ± SD. Immunoassay data are presented as mean ± standard error.

3. Results

Data from two of the initial nine participants were excluded from the analyses. One participant could not complete the HIIT protocol. Hemolysis impeded cytokine measurements of the second participant's samples.

There were no significant changes in the plasma levels of CRP, IL-1β, IL-6, IL-10, and IGF-1 from baseline to either 30 min or 2 days after the intervention (Table 2). However, there was a significant decrease in the IL-6/IL-10 ratio from baseline to thirty minutes after ET (−20%; p = 0.047). MCP-1 concentrations also decreased significantly from baseline to 2 days after ET (−17%); p = 0.03). These changes reflected at the 30 min mark where a trend towards a significant difference between the post-END and post-HIIT change in CRP (ET 3.29 ± 1.01 > HIIT 2.07 ± 0.69; p = 0.1) and IL-6 (ET 27.25 ± 19.38 < HIIT 37.76 ± 19.49; p = 0.09) was observed. A significant correlation between CEP and a calculated score based on IL-1β and IL-6 levels (Pearson R = 0.63; p = 0.01) was also observed. The response to the two cytokines is cooperative or synergistic and was calculated using normalized values of all patients from all points in time and an appropriate model for the synergistic action of IL-6 and IL-1β as suggested by Ganter et al. [9].

4. Discussion

This is the first study to directly compare the inflammatory response to single-bout ET and HIIT in the same cohort as well as investigate acute-phase response and medium-term response of cytokines to single-bout HIIT and ET. Our cohort was comparable in numbers to previous publications but used a repeated-measures design to obtain a better statistical power.

Endurance training reduces MCP-1 levels and has been associated with a reduction in the development of atheroscle-rosis, metabolic syndrome, and diabetes by acting on visceral fat reserves. MCP-1 has also been shown to be reduced following moderate levels of exercise in heart failure patients [20, 21]. Therefore, we sought to determine whether MCP-1 is reduced in young adults undertaking HIIT and whether
this differs to results reported for ET. MCP-1 concentrations decreased after both training sessions and significantly decreased two days after ET. Zwartsoot et al. [8] showed that MCP-1 levels were significantly elevated immediately after exercise and Sugama et al. [22] demonstrated a high flush-out rate of this protein into urine. Therefore, our measured decreased levels may be lower following ET and HIIT due to the washout effect. However, the decreased MCP-1 levels two days after ET and after HIIT may also be due to a long-term decline in oxidative stress [23]. MCP-1 levels continued to decrease further following HIIT, which may be due to a more effective reduction in oxidative stress in addition to reported increased levels of lactate acid following HIIT [24, 25]. In the context of beneficial effects of single-bout ET and HIIT, one major health benefit of maintaining generally low levels of MCP-1 is the reduction of risk of cardiovascular disease and diabetes [26].

Our initial hypothesis that inflammatory responses would be different following single-bout ET versus single-bout HIIT was confirmed by a trend towards a greater decrease in the proinflammatory CRP and an increase in IL-6 levels following HIIT at thirty minutes following exercise. Neither training protocol appeared to cause significant acute changes of any single inflammatory cytokine possibly due to the relatively short training period (HIIT: 25 min, ET: 45 min). Previous investigations with shorter single-bout exercise duration (30 min) and lower intensity (50% maximal oxygen uptake) reported a comparable inflammatory response [12].

Previous work by Kasapis and Thompson [27] reported consistent increases in CRP levels after very strenuous exercise including marathons. The CRP decrease after HIIT in the present study is in agreement with the findings of Hovanloo et al. [6] who demonstrated a small CRP decrease 48 h after completing six HIIT sessions at a less strenuous level compared to Kasapis and Thompson.

A slight post-HIIT increase in IL-6 levels, which is in agreement with much of the literature, was observed [3, 8, 28]. These nonsignificant increases may be due to the short duration or lower exercise intensity, which did not affect muscle physiology enough to lead to a significant increase in IL-6 levels [29]. Single-bout ET and HIIT therefore have minor effects on muscle tissue integrity that nonetheless may lead to a greater reduction in CRP levels following HIIT by IL-6 acting as proinflammatory cytokine [1].

IL-6 is also the main inducer of CRP transcription but requires the presence of sufficient IL-1β in this regard. Although the presence of IL-6 by itself significantly raises CRP transcription, maximal expression only occurs when both cytokines are active [9]. Since IL-1β levels decreased after HIIT, which is in agreement with previous findings by Zwartsoot et al. [8], we employed a mathematical model to simulate a synergistic action of IL-6 and IL-1β on CRP expression as previously suggested by Ganter et al. [9]. The regulatory role of IL-1β in this context helps to explain why post-HIIT CRP levels decreased in spite of elevated IL-6 levels in our study. However, future studies will be required to validate this model.

No significant change in IL-10 levels was observed, although a slight decrease was noted, which is in agreement with the findings of Hovanloo et al. [6] and Zwartsoot et al. [8] regarding HIIT. Our findings for the ET intervention are inconsistent with the literature as most studies reported marked postexercise increases of IL-10 in contrast to our study showing no effect [22, 30]. However, these followed much more exhausting bouts of exercise. The recent study by Markovitch et al. [12], which resembles our ET protocol more closely, did not detect a significant IL-10 change. The observed decrease of plasma IL-10 levels could be attributed to a similar mechanism found for the MCP-1 decrease as Sugama et al. [22] also reported a rapid increase of IL-10 in the urine 1.5 h after exercise.

Although mean IGF-1 levels decreased after both ET and HIIT, there was no significant change to be noted. The findings are consistent with the results by Nindl et al. [14] who demonstrated that IGF-1 levels do not increase or decrease significantly after moderate or long duration aerobic or anaerobic exercise. The present study adds to this body of evidence and shows that circulating total IGF-1 levels do not change significantly after single bouts of ET or HIIT.

Regular aerobic exercise such as ET improves general health. HIIT, however, can produce similar results to ET including muscle oxidative capacity and mitochondrial enzyme activity, but in addition to providing various cardiometabolic benefits HIIT is also very time-efficient [17]. The lack of large-scale post-HIIT or post-ET inflammatory responses indicates that a regimen of two to three HIIT or ET sessions per week may noticeably aid in improving general health and fitness such as body fat percentage, maximal oxygen uptake, and cardiovascular endurance while exerting relatively little stress on the immune system in untrained and healthy young adults [16].

5. Conclusions

The novel findings of the present study are that (1) there was no significant difference between HIIT and ET in regard to individual cytokine responses and (2) IL-6 may be acting as an anti-inflammatory cytokine that leads to a greater decrease in CRP following HIIT and similar to ET a decreased MCP-1. These results support the hypothesis that HIIT may present a valid alternative to ET for individuals that are short on time or personally prefer a HIIT-type regimen to achieve equivalent overall health benefits to ET.

Additional Points

Practical implications are as follows:

(i) Lifestyle-induced sedentaryness is a major contributor to obesity and diabetes in western societies and efforts to increase general physical activity are mostly unsuccessful. Therefore, various exercise protocols should be assessed.

(ii) The present study shows that there is no significant difference between the inflammatory responses to conventional endurance training and high-intensity
interval training and that neither protocol caused severe acute cytokine increases.

(iii) Time-efficient high-intensity interval training, therefore, should be considered a valid alternative to conventional training approaches to improve general health and fitness.

Competing Interests
The authors declare that they have no competing interests.

Acknowledgments
The authors thank all volunteers for their participation and Hoffmann-La Roche for providing the blood glucose strips and acknowledge the highly valuable contribution of Simon McDonald in conducting the nonparametrical statistical analyses. Helpful suggestions on a previous draft were made by Dr. Ian Spence.

References

