ASSOCIATION OF OXIDATIVE STRESS AND INFLAMMATION WITH ALTERED HAEMORHEOLOGY IN METABOLIC SYNDROME

BY

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

February, 2015.

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Certificate of Authorship

I Prajwal Gyawali hereby declare that this submission is my own work and that, to the best of my knowledge and belief, 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 acknowledgment 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.

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................................        ....................
Signature         Date

x
Dedicated
To
My wife: Pinkey Timilsina
My parents: Shiva Gyawali and Kalpana Gyawali

Acknowledgement
I would like to express my special appreciation and thanks to my PhD supervisors: Dr. Ross S Richards (principal supervisor), Dr. Paul Tinley, Dr. Ezekiel Uba Nwose and Dr. Phillip T Bwititi for their aspiring guidance and constructive suggestions throughout the project. This work would not have been possible without the Endeavour Postgraduate scholarship. I am using this opportunity to express my gratitude to Australian government and Scope Global for providing me with Endeavour Scholarship.

I would also like to thank Kylie Kent, Cheryl Kolbe, Amanda Eddy and Lisa Thompson for all the technical support they offered to me during the participants’ recruitment and laboratory analysis. My sincere thanks also goes to Wesley Ward, Sylvia McAra, Caroline Robinson, Lyn Ewart and the support staff of ‘Community Engagement and Wellness Centre’ for helping me to recruit the volunteers. Special thanks to my team mate Diane Hughes, who as a good friend, was always willing to help and give her best suggestions. It would have been a lonely lab without her. I am grateful to Greg Fry for his help with endnotes and bibliographies.

I would specially like to express the deepest appreciation to all the research participants who agreed to participate voluntarily in my research for the betterment of science.
Ethics Approval

This research was approved by the Human Research Ethics Committee (HREC), Charles Sturt University (CSU) with protocol number 2012/131 (Appendix 1).
Abstract

**Background:** In carrying out their role of free radical scavenging, erythrocytes become damaged due to oxidation of membrane lipids and proteins. Such damage changes the morphology and rheological properties (erythrocyte aggregation, erythrocyte deformability and whole blood viscosity) of the erythrocytes. It has been shown that altered haemorheology leads to slowed coronary blood flow, resulting in altered microcirculation. Erythrocyte aggregates may form a thrombus at low shear stress. Vascular dysfunction is associated with metabolic syndrome and its components. In this context, the objective of our study was to demonstrate haemorheological abnormalities and decreased peripheral blood flow in metabolic syndrome and to associate abnormal blood rheological characteristics with chronic inflammation and oxidative stress in the same subjects.

**Materials and methods:** 100 participants were recruited from a rural Australian town (Albury and Wodonga). Anthropometric measurements (blood pressure from arm, waist circumference, height and weight) and twenty mL of blood sample were obtained from the research participants. Blood pressure was also measured from the great toe of the research participants (to calculate toe brachial pressure index) using the Systoe method to assess the status of peripheral blood circulation. Whole blood viscosity, erythrocyte aggregation, erythrocyte deformability, erythrocyte morphology, lipid profile and blood sugar level were analysed. In addition, oxidative stress markers: erythrocyte reduced glutathione, superoxide dismutase and urinary isoprostanes; inflammatory markers high-sensitivity C-reactive protein; and thrombotic marker D-dimer were measured. Recruited participants were classified into two groups: with and without metabolic syndrome following the National Cholesterol Education Program Adult Treatment Panel III
definition (Expert Panel on Detection Evaluation Treatment of High Blood Cholesterol in Adults, 2001). Data were analysed by IBM SPSS 20 software.

Results: Whole blood viscosity ($P$-value <0.0005) and erythrocyte aggregation ($P$-value <0.0005) were higher whereas erythrocyte deformability ($P$-value <0.0005) was lower in participants with metabolic syndrome when compared to participants without metabolic syndrome. Morphologically abnormal erythrocytes (when viewed using scanning electron microscopy) were found to be significantly higher in metabolic syndrome compared to non metabolic syndrome group ($P$-values for: acanthocytes <0.0005, echinocytes 0.006 and stomatocytes 0.001). Altered haemorheology and abnormal erythrocyte morphology were significantly associated with chronic inflammation and oxidative stress in metabolic syndrome. Also, there was a significant reduction in peripheral blood flow (decreased toe brachial pressure index, $P$-value <0.0005) among metabolic syndrome participants.

Conclusions: Haemorheological alterations and reduced peripheral blood flow were demonstrated in metabolic syndrome. Defective microcirculation due to altered haemorheology could be one of the several pathogenic mechanisms underlying complications of metabolic syndrome. Alterations of haemorheology in metabolic syndrome are probably due to the effect of chronic inflammation and oxidative stress. Free radicals and inflammatory molecules generated in increased concentration in metabolic syndrome seem to damage the erythrocyte changing its morphology and deformability which affect other haemorheological parameters. The negative effect of inflammatory and oxidant molecules seen in the cardiovascular system could be due to
the resulting altered haemorheology. Altered haemorheology could be the bridge that connects metabolic syndrome with cardiovascular diseases.

Manuscript published from the study


2) Gyawali P, Richards RS. Association of altered hemorheology with oxidative stress and inflammation in metabolic syndrome. Redox report 2014, DOI: http://dx.doi.org/10.1179/1351000214Y.0000000120


8) Gyawali P, Richards RS, Bwititi PT, Tinley P, Nwose EU. Association of oxidative stress and inflammation with non-invasive peripheral arterial disease markers in participants with and without metabolic diseases. Vascular medicine 2015. (Under review)
List of abbreviations

ABPI: Ankle brachial pressure index
BMI: Body mass index
BPT: Blood passage time
CVDs: Cardiovascular diseases
DALI: Direct absorption of lipoproteins
DM: Diabetes mellitus
EI: Elongation index
EI_{max}: Elongation index maximum
FFA: Free fatty acid
FBG: Fasting blood glucose
GSH: reduced glutathione
HDL: High density lipoprotein cholesterol
hs-CRP: high sensitive C-reactive protein
IDF: International Diabetes Federation
IL-6: Interleukin-6
IRS: Insulin receptor substrate
LDL-C: Low-density lipoprotein cholesterol
MetS: Metabolic syndrome
NCEP ATP III: National Cholesterol Education Programme Adult Treatment Panel III
NMR: Nuclear magnetic resonance
PAD: Peripheral arterial diseases
PI: Phosphatidyl inositol
PKC: Protein kinase C
SOD: Superoxide dismutase
SS_{1/2}: Shear stress (required for the half of maximal deformation)
TBPI: Toe brachial pressure index
TNF: Tumour necrosis factor
WBV: Whole blood viscosity
WHO: World Health Organization
Chapter 1: Introduction

1.1 Background

Metabolic syndrome (MetS) consists of multiple, interrelated risk factors of metabolic origin that appear to promote the development of cardiovascular diseases (CVDs) (Expert Panel on Detection Evaluation Treatment of High Blood Cholesterol in Adults, 2001). It is often a precursor for identifying individuals at high risk of type 2 diabetes mellitus (type 2 DM) and CVD (Alberti, Zimmet, & Shaw, 2005). The Australian Diabetes, Obesity and Lifestyle Study reported that the prevalence of MetS in the Australian population aged 25 years and older was 30.7% in 2007 (Cameron, Magliano, Zimmet, Welborn, & Shaw, 2007). This figure could now be higher owing to the fact that the prevalence of obesity and DM is dramatically increasing in Australia (Walls et al., 2012). Obesity, high blood pressure, impaired glucose tolerance and dyslipidaemia are included in MetS definitions given by different organisations (Alberti, Zimmet, & Shaw, 2006; Alberti & Zimmet, 1998; Expert Panel on Detection Evaluation Treatment of High Blood Cholesterol in Adults, 2001). The International Diabetes Federation (IDF) has suggested that oxidative stress, chronic inflammation and prothrombotic state also be included in research of MetS, since these components are considered to be closely associated with MetS (Alberti et al., 2006).

Oxidative stress is increased in MetS (Dandona, Aljada, Chaudhuri, Mohanty, & Garg, 2005; Hansel et al., 2004), and erythrocytes play a significant role in scavenging the free radicals generated in MetS. It has been proposed that in carrying out their role of free radical scavenging, erythrocytes become damaged due to oxidation of their membrane lipids and proteins, and furthermore, that the oxidation consumes endogenous reducing substances (Richards, Roberts,
McGregor, Dunstan, & Butt, 1998). Such damage may increase the rigidity of the erythrocyte membrane, changes its shape, and reduce its deforming capacity (Madsen, Nagel, Rybicki, & Schwartz, 1991; Simmonds et al., 2011). Under physiologic conditions, deformability allows erythrocytes of 7 μm diameter to traverse through capillaries with diameters no more than 3-5 μm, thus facilitating tissue oxygenation. Hence, reduced deformability results in impaired perfusion and oxygen delivery in peripheral tissues (Yedgar, Koshkaryev, & Barshtein, 2002). Furthermore, alterations in shape and deformability due to oxidative damages, and the increased concentration of inflammatory molecules in the blood in MetS all contribute to a favorable environment for erythrocyte aggregation and an associated increase in blood viscosity. Thus, there is potential for gross alterations of haemorheological properties in MetS due to oxidative stress and chronic inflammation.

Altered haemorheology has been shown to negatively affect microcirculation (Baskurt & Meiselman, 2003; Konstantinova et al., 2004; Pirrelli, 1999; Sandhagen, 1999), and microvascular abnormalities affect pressure and flow of blood (Levy, Ambrosio, Pries, & Struijker-Boudier, 2001; Serné, de Jongh, Eringa, IJzerman, & Stehouwer, 2007). Vascular dysfunction is associated with MetS and its components (Czernichow et al., 2010; Serné et al., 2007; Vykoukal & Davies, 2011). Similarly, arterial stiffness and a concomitant reduction in blood flow have been associated with unfavourable rheology (Satoh et al., 2009). Such evidence suggests that alterations in haemorheology in MetS along with increases in oxidative stress and chronic inflammation could concertedly affect the blood vessels, vascular properties and flow of blood.
1.2 Objectives

There is the propensity for erythrocytes to be involved in cellular oxidative stress, and thus, for them to be a common factor in abnormalities of MetS. Furthermore, alterations in rheology possibly due to the generation of oxidative stress and chronic inflammation could negatively affect the peripheral blood flow. In this context, the objectives of the present study are to assess the levels of oxidative stress and chronic inflammation in participants with MetS and to compare them with those in participants without MetS. Also, the objective of the present study is to demonstrate altered haemorheology in participants with MetS when compared to those without MetS and determine if there is an association between oxidative stress and inflammation levels with altered haemorheology in participants with MetS. Finally, the study also aims to discover the status of peripheral arterial diseases (PAD) by using the non-invasive marker of peripheral blood flow in participants with MetS and compare it with the status of peripheral blood flow in participants without MetS.

1.3 Research Hypotheses

The following research hypotheses are tested in the present study to satisfy the goals and objectives of the study.

1) Haemorheological parameters are unfavourably altered in MetS when compared to healthy controls.

2) Alterations of haemorheological parameters in MetS are associated with oxidative stress and inflammation generated in MetS.
3) MetS is more strongly associated with haemorheological alterations than with novel cardiovascular risk factors: oxidative stress, inflammation and non-invasive markers of PAD.

The objectives of the study and the corresponding results and discussion section in the present study are shown in Table 1.1.

Table 1.1: Table showing the objectives of the study with its corresponding results and discussion section

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4.7 Peripheral vascular disease markers: ABPI and TBPI and its association with haemorheology, oxidative stress and chronic inflammation

5.6 Association of oxidative stress and chronic inflammation with peripheral vascular diseases markers: ABPI and TBPI

7. Compare the association/relationship between haemorheological parameters, oxidative stress, chronic inflammation and reduced peripheral blood flow in participants with and without MetS

4.8 Association of altered haemorheology, peripheral vascular diseases markers and novel cardiovascular risk factors with metabolic syndrome

5.7 Comparison of the association of altered haemorheology, peripheral vascular diseases markers and novel cardiovascular risk factors with metabolic syndrome

5.8 Clinical significance of the study: Altered haemorheology and CVDs

1.4 Rationale of the study

After an extensive review of literature on MetS, it was revealed that there is still (i) a lack of certainty regarding its pathogenesis, (ii) considerable doubt regarding its value as a CVD risk marker, and (iii) no information to warrant its designation as a syndrome (Kahn, Buse, Ferrannini, & Stern, 2005). The mechanisms underlying the link between MetS and CVD need further investigation (Alberti et al., 2006). There is a clear need for an updated description of the pathophysiology of MetS and it is strongly rationalised that a description of the pathophysiology of MetS is incomplete without referring to the haemorheology.

The aim of this study is to investigate the hypothesis that haemorheology is altered in MetS. The present study also aims to compare any possible association of haemorheology and other novel cardiovascular risk factors such as oxidative stress, chronic inflammation and decreased peripheral blood flow with MetS. If altered haemorheology is associated with MetS, and if this
association is found to be stronger than that between MetS and other novel cardiovascular risk factors, then it could be suggested that the altered haemorheology is another important mechanism that contributes to the morbid biology of MetS. Haemorheological parameters may be altered by the effect of oxidative stress and chronic inflammation. The morphological changes to erythrocytes could be a consequence of oxidative stress. Vasculopathy and tissue deoxygenation may occur as a consequence of altered haemorheology and these play a major role in the development of CVD. Hence, altered haemorheology could be the bridge that links MetS with CVD. Altered haemorheology could be targeted to reduce CVD and become a potential therapeutic focus for intervention. Hence, findings from this study could help in better understanding MetS pathogenesis. Also, incorporation of the rheological components into the definition of MetS will increase the diagnostic value of MetS.

1.5 Research approach

There are three dimensions to the study:

1) Oxidative stress and chronic inflammation: This study aims to demonstrate the increased oxidative stress and chronic inflammation in MetS group compared to non-MetS group. Oxidative stress and chronic inflammation are the novel cardiovascular risk factors and the research components of MetS (Alberti et al., 2005). Biomarkers of oxidative stress and chronic inflammation are measured in the present study. Oxidative stress is measured by the markers erythrocyte reduced glutathione (GSH), erythrocyte superoxide dismutase (SOD) and urinary 15-isoprostanes F_{2t}. Chronic inflammation is measured by high sensitivity C-reactive protein (hsCRP) and the prothrombotic marker, D-dimer. These biomarkers provide information on oxidative

2) Haemorheology: The present study aims to study the haemorheological profile in participants with and without MetS and associate the haemorheological profile with oxidative stress and chronic inflammation. Erythrocyte haemodynamic properties that determine blood flow are self-aggregability, deformability, morphology and whole blood viscosity (WBV) (Stoltz, Singh, & Riha, 1999a; Yedgar et al., 2002). Erythrocytes need to change shape to pass through the capillary bed and oppose the flow resistance, and this is directly related to their deformability (Chien, 1987). Haemorheological factors that affect the blood flow are measured in the present study.

3) Evaluation of PAD: Peripheral arterial disease is common in MetS. Measurement of peripheral blood flow is commonly used to diagnose PAD. The indeces of PAD has been associated with oxidative stress and chronic inflammation in the present study. Reductions in blood flow indicate the possibility of atherosclerotic lesions, and thus, suggest the occurrence of PAD (Brooks et al., 2001). Assessment of PAD in the present study is done by two non-invasive techniques: 1) Ankle Brachial Pressure Index (ABPI) and 2) Toe Brachial Pressure Index (TBPI).
Chapter 2: Literature review

Abstract

This chapter presents a review on MetS and its effect on cardiovascular health. The epidemiological data highlights the worldwide epidemic of obesity, insulin resistance, and MetS. Insulin resistance is associated with oxidative stress, and the literature suggests that oxidative stress is the prior event of MetS and that it affects the progression of the syndrome. Oxidative stress has also been associated with alterations in haemorheology. The concept of haemorheology has been explained in detail in the present review. The interplay of haemorheological factors, oxidative stress and inflammation has a detrimental effect on MetS due to the gross disturbance in microcirculation. This chapter deals with MetS and insulin resistance and provides a review of the haemodynamic aspect of MetS by associating components of MetS along with oxidative stress and chronic inflammation with haemorheological variables. The summary of the changes of altered blood rheology in different disease states have been provided in tables. At the end this chapter, the use of an ABPI and a TBPI in determining peripheral blood flow and peripheral vascular diseases is highlighted.
Chapter outline

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2.6 Summary of literature review
2.1 Concept of Metabolic syndrome (MetS)

In 1988, Reaven noted that several risk factors such as, dyslipidaemia, hypertension and hyperglycaemia commonly cluster together (Reaven, 1988). This clustering was called Syndrome X, and it was recognised as a multiplex risk factor for cardiovascular diseases (CVDs). Reaven postulated that insulin resistance underlies Syndrome X (hence the commonly used term insulin resistance syndrome). Later, the term ‘metabolic syndrome’ was used for this clustering of metabolic risk factors (Alberti et al., 2005).

MetS has received increased attention in the last two decades (Kahn et al., 2005). It consists of multiple, interrelated risk factors of metabolic origin that appear to promote the development of CVD (Jiang et al., 2014). MetS has been defined by various organisations such as:

- World Health Organization (WHO)
- National Cholesterol Education Programme Adult Treatment Panel III (NCEP ATP III)
- International Diabetes Federation (IDF)
- European Group for the Study of Insulin Resistance
- National Heart, Lung and Blood Institute/American Heart Association and
- American Association of Clinical Endocrinologist.

Multiple attempts have been made to define MetS as new information has become available with respect to its role in cardiovascular health. The WHO first attempted to define the syndrome in 1998, but new research and evidence accumulated since then made it necessary for other organisations to develop different definitions with a view to improve prediction of CVDs.
2.1.1 Significance of MetS

MetS is not an absolute risk indicator, because it does not contain many of the factors that determine absolute risk (e.g. sex, cigarette smoking, and low density lipoprotein cholesterol (LDL-C) levels). Patients with MetS are at twice the risk of developing CVD over the next five to ten years as individuals without the syndrome, and the risk over a lifetime is undoubtedly higher (Lakka et al., 2002). Furthermore, MetS confers a five-fold increase in risk for type 2 DM (Alberti et al., 2009). Hence, the main significance of MetS is its association with an increased risk of DM (Grundy, Hansen, Smith, Cleeman, & Kahn, 2004) and CVD (Isomaa et al., 2001; Lakka et al., 2002).

2.1.2 Underlying causes of MetS

Disorders of adipose tissue and obesity; insulin resistance; and molecules of hepatic, vascular and immunologic origin have been proposed as the aetiological factors of MetS (Grundy, Brewer Jr, Cleeman, Smith Jr, & Lenfant, 2004). The most accepted hypothesis to describe the pathophysiology of MetS pertains to insulin resistance (Eckel, Grundy, & Zimmet, 2005). The insulin resistance, in turn, is caused by obesity (Booth, Magnuson, & Foster, 2014). A plentiful supply of inexpensive food and sedentary life style are the major causes for increased obesity (Grundy, 2004). Therefore, unhealthy eating habits and lack of exercise seems to be the underlying cause of MetS. Insulin resistance and the effect of obesity on insulin resistance has been discussed in detail in the section 2.2.1 and 2.2.2.
2.1.3 Critical evaluation of the MetS definition

The American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) raised several questions regarding the concept of MetS based on a critique of the earlier WHO and Adult Treatment Panel III criteria (Kahn et al., 2005). Their major concerns were:

1. whether it is a syndrome in view of the fact that its exact aetiology is unknown
2. whether it serves a useful purpose in managing and treating patients
3. the unnecessary labelling, and thus, medicalising of people according to the definition of the syndrome.

The authors also argue that most of the features of MetS are weighted towards DM and CVD, and when these two markers are eliminated from the definition, the prognostic significance of the syndrome is lost (Kahn et al., 2005). It has also been suggested that the concept of MetS has been largely driven by the pharmaceutical industry to market the products (Gale, 2005). There is no specific clinical management strategy for people with MetS. The individual components of MetS, such as hypertension, dyslipidaemia and DM, are treated separately through their own specific guidelines (Gale, 2005). Hence, there is no benefit in keeping these components under a single umbrella, because MetS is not more important than the sum of its parts (Gale, 2005). The IDF MetS definition working group (Alberti et al., 2006) has acknowledged that there are many unanswered questions relating to MetS. They agreed that the best and most predictive definition of MetS and its components has not been identified, and that the aetiology of MetS is still unclear. The current study will investigate the possible association of MetS with altered
haemorheology. The study also considers the two important dimensions of MetS: oxidative stress and chronic inflammation. Our study perhaps will address some of the unanswered queries related to the pathophysiology of MetS.

### 2.1.4 Comparison of definitions of the MetS

The definitions given by WHO, NCEP ATP III and IDF have been widely used in the study of MetS and predominate in the literature. Hence, the definitions given by these organisations are highlighted in this review.

Table 2.1: Comparison of the definitions of MetS

<table>
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<tr>
<td>Compulsory criteria</td>
<td>Type 2 DM or impaired fasting glucose or impaired glucose tolerance or insulin resistance (hyperinsulinaemic, euglycaemic clamp-glucose uptake in lowest 25%)</td>
<td>Central obesity defined as a waist circumference with ethnic specific cut-off values</td>
<td>No compulsory criteria</td>
</tr>
<tr>
<td>Obesity</td>
<td>Plus any two of the following</td>
<td>Plus any two of the following</td>
<td>Any three or more of the following</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Body mass index (BMI) &gt; 30 kg/m² or waist:hip ratio &gt;0.9 in men, &gt;0.85 in women</td>
<td>Blood pressure ≥ 130/85 mm Hg or in</td>
<td>Blood pressure ≥ 130/85 mm Hg or in</td>
</tr>
</tbody>
</table>

14
<table>
<thead>
<tr>
<th><strong>High density lipoprotein – cholesterol (HDL-C)</strong></th>
<th>medication</th>
<th>medication</th>
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<tr>
<td>&lt;0.9 (male) or &lt;1.0 (female) mmol/L</td>
<td>&lt;1.0 mmol/L (male), &lt;1.3 mmol/L (female) or specific treatment</td>
<td>&lt;1.0 mmol/L (male), &lt;1.3 mmol/L (female) or specific treatment</td>
</tr>
<tr>
<td><strong>Triglyceride</strong></td>
<td>≥1.7 mmol/L</td>
<td>≥1.7 mmol/L or specific treatment</td>
</tr>
<tr>
<td><strong>Fasting plasma glucose</strong></td>
<td>See compulsory criteria Glucose ≥6.1 mmol/L</td>
<td>Glucose ≥5.6 mmol/L or previously diagnosed DM</td>
</tr>
<tr>
<td><strong>Microalbuminuria</strong></td>
<td>Microalbuminuria: albumin excretion ≥20 µg/min</td>
<td>-</td>
</tr>
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The general components of the MetS common to all the three definitions (WHO, NCEP ATP III and IDF) are:

- obesity/abdominal obesity
- increased blood pressure
- insulin resistance/ glucose intolerance
- dyslipidaemia
- prothrombotic state
- proinflammatory state.
2.1.5 Contrasting features of the multiple definitions

1) In the WHO definition of MetS, insulin resistance is a required component for diagnosis (Alberti & Zimmet, 1998). Insulin resistance is defined as one of the following: type 2 DM, impaired fasting glucose, impaired glucose tolerance, or for those with normal fasting glucose values (≤6.1), glucose uptake below the lowest quartile for the background population under hyperinsulinemic, euglycemic conditions. The special testing of glucose status beyond routine clinical assessment is necessary to diagnose MetS.

2) A higher blood pressure is required in the WHO definition than in either the ATP III or IDF definitions (Alberti & Zimmet, 1998).

3) As a component of obesity, Body Mass Index (BMI) (or increased waist:hip ratio) is used by WHO instead of waist circumference (as used by NCEP ATP III and IDF). Central obesity, as assessed by waist circumference, is essential component in the IDF definition.

4) Microalbuminuria is listed as one criterion in the WHO definition, but not in the other definitions.

2.1.6 Confusion due to multiple definitions

The existence of multiple definitions for MetS has inevitably led to confusion and many studies and research papers have compared the definitions (Cameron et al., 2007). It is not possible to make comparisons between the data from studies when different definitions have been used to identify patients with MetS. Just as the prevalence of the individual components of MetS varies between populations, so too does the prevalence of MetS itself. There are certain important
differences among these definitions. For instance, the ATP III definition has a lower diagnostic threshold than the WHO definition for certain characteristics (i.e. high density lipoprotein cholesterol (HDL-C) and hypertension) and a higher threshold for others (i.e. obesity).

2.1.7 Prevalence of MetS in Australia

The prevalence of MetS in the Australian population has been reported to range between 21.7% and 30.7% depending on whether the WHO, modified NCEP ATP III or IDF definition is applied (Cameron et al., 2007). Similarly, among the non-diabetic population, the prevalence of MetS using the European Group for the Study of Insulin Resistance definition was reported as 13.4% (Cameron et al., 2007). The AusDiab steering committee has reported the prevalence of MetS to be 25% in males, and 14% in females (Dunstan et al., 2005). The current (i.e. 2014) prevalence of MetS has not been reported, but is most likely to have increased owing to the fact that the incidences of obesity and diabetes (factors that are associated with MetS) are increasing in Australia (Walls et al., 2012).
Section summary: Several authorities have provided the definition of MetS. They all differ from one another with respect to certain aspects; however, they all contain the key features of obesity, dyslipidaemia, insulin resistance and hypertension. The most significant feature of MetS is its association with the cardiovascular event. The incidences of obesity and DM are increasing at an alarming rate in Australia, and hence, a high prevalence of MetS has been reported. However, an extensive review of the literature on MetS revealed that there is a lack of certainty regarding its pathogenesis, and considerable doubt regarding its value as a CVD risk marker. Some authors argue that critically important information is still needed to warrant its designation as a syndrome. The effect of MetS on cardiovascular system could be due to the generation of oxidative stress in MetS, or due to the insulin resistance responsible for driving the MetS itself. This study focuses on the effect of MetS on red blood cells and the association of altered red blood cells with vascular disease. The next subchapter highlights the mechanism of insulin resistance, and associates oxidative stress with insulin resistance and DM complications.
2.2 Insulin resistance, oxidative stress and MetS

2.2.1 Insulin resistance

Insulin resistance is defined as a subnormal biological response to a given concentration of insulin (Moller & Flier, 1991). Though insulin has multiple functions, the term ‘insulin resistance’ is often used in relation to glucose homeostasis. Since oxidative stress, insulin resistance, chronic inflammation and MetS are interrelated biochemically and pathologically, they are discussed together. This part of the literature review covers the following topics:

- obesity/inflammation and insulin resistance
- free fatty acids (FFA) and insulin resistance
- oxidative stress and insulin resistance
- oxidative stress and DM complications.

2.2.2 Obesity/inflammation and insulin resistance

Insulin resistance is generally considered a major pathophysiologic link between obesity and type 2 DM (Hardy, Czech, & Corvera, 2012; Kahn, Hull, & Utzschneider, 2006). Adipose tissue is the body’s largest reserve of fuel, storing energy in the form of triglycerides. This energy can be rapidly mobilised during starvation and in other times of need (Flatt, 1995). The switch from energy storage to mobilisation within adipocytes is regulated by hormonal signals from other tissues and organs, including the pancreas (insulin), the sympathetic nervous system (catecholamines) and the adrenal glands (glucocorticoids). Apart from its role in fueling homeostasis, adipocytes express and secrete numerous peptide hormones and cytokines, including tumour necrosis factor (TNF)-α; plasminogen-activator inhibitor-1, which helps in
maintaining haemostasis; angiotensinogen, whose proteolytic product regulates vascular tone; and leptin, which plays a central role in regulating energy balance (Siiteri, 1987). These products may be an additional link between adipose function or mass, and insulin resistance (Dandona, Aljada, & Bandyopadhyay, 2004).

TNF-α is a cytokine produced mostly by macrophages and adipose tissues. Production of excess TNF-α by human adipose tissues in obesity has been linked to insulin resistance (Hotamisligil, Arner, Caro, Atkinson, & Spiegelman, 1995). Northern blot analysis of subcutaneous fat tissue demonstrated that the TNF-α m-RNA is endogenously present in human adipose tissue. The concentration of TNF-α m-RNA expression was elevated in adipose tissues from obese individuals compared with those in age-matched lean individuals (Hotamisligil et al., 1995). The study suggested that elevated m-RNA expression in obesity was specific for TNF-α, and that detection of this cytokine was not due to generalised expression of cytokine genes in adipose tissues in obesity, since TNF β, IL 1β and IL 6 mRNA were not detected (Hotamisligil et al., 1995). TNF-α m-RNA expression was significantly and positively correlated with BMI, fasting insulin level and fasting triglyceride level. In contrast, no significant correlation was observed between TNF-α expression and total cholesterol concentration, waist:hip ratio and age of the patients (Hotamisligil et al., 1995). In apparently healthy patients, significantly reduced fasting serum insulin levels (43.1±5.1 to 26.2±3.5 µU/ml, P = 0.001) and TNF-α-mRNA expression were noticed after weight reduction. TNF-β, IL-1β and IL-6 m-RNA expressions were not observed either before or after weight reduction. These results are suggestive of the role of TNF-α in insulin resistance (Hotamisligil et al., 1995). It was demonstrated in an animal model that TNF-α-induced insulin resistance was due to the inhibition of insulin-stimulated
autophosphorylation of the insulin receptor and phosphorylation of insulin receptor substrate 1 (IRS-1) \textit{in vivo} (Hotamisligil, Budavari, Murray, & Spiegelman, 1994). Reduction of kinase activity in the insulin receptor was not observed in obese non-diabetic patients. Hotamisligil et al. (1995) suggested that TNF-\(\alpha\) induced insulin resistance may involve regulation of glucose transporter number or activity, or modulation of steps downstream of insulin receptor and IRS-1 in the signal transduction cascade.

2.2.3 Free fatty acids (FFA) and insulin resistance

Generation of one or more metabolic messengers by the adipose tissue in obese individuals would inhibit insulin action on muscle and/or the liver. Boden (1997) suggested FFA as one of these messengers, which are increased in obese individuals due to a high rate of lipolysis from expanded muscle mass. He proposed that in the development of type 2 DM, FFA eventually fails to stimulate insulin secretion (Boden 1997). Hepatic/muscle insulin resistance is often left unchecked, resulting in hepatic overproduction or muscle under-utilisation of glucose (figure 2.1).

FFA-induced insulin resistance serves an important physiological role in preserving glucose for oxidation in the central nervous system when glucose is scarce, like during periods of fasting or prolonged exercise. These same mechanisms in obesity can become counterproductive by inhibiting glucose utilisation when there is no need to spare glucose (Boden, 1997). Most obese individuals have normal glucose tolerance, because their insulin resistance is in parallel with enhanced insulin secretion. The exact mechanism of enhanced insulin secretion in euglycaemic obese state is unclear. It was suggested that liver insulin uptake is reduced due to exposure to elevated concentration of FFA coming from large intra-abdominal fat depots (Boden, 1997).
Figure 2.1 Conceptual model of Boden (1997): Boden (1997) proposed that increased free fatty acid in obese individuals genetically predisposed to develop type 2 DM, fails to stimulate insulin secretion. Peripheral glucose underutilisation and hepatic glucose overproduction leads to type 2 DM.

Randle et al. (Randle, Priestman, Mistry, & Halsall, 1994), introduced the concept that FFA interferes with glucose utilisation and suggested that increased acetyl CoA production in mitochondria inhibits pyruvate dehydrogenase, which is rate-limiting for glucose oxidation (Randle et al., 1994). The resultant increased citrate in the cytoplasm was proposed to down-regulate phosphofructokinase and glycolytic flux. In addition, elevated glucose-6-phosphate inhibits hexokinase and glucose phosphorylation (Randle et al., 1994). Randle et al. (1994) hypothesised that the availability of lipids as a source of fuel triggers metabolic signals that
impair the use of glucose through inhibition of the key glycolytic enzymes. This mechanism alone is not sufficient to describe the effect of FFA on carbohydrate metabolism in obesity. The *in-vivo* inhibition of glucose transport or phosphorylation after three to four hours of fat infusion could be another of the several mechanisms by which FFA inhibits insulin-stimulated glucose uptake. Muscle glycogen synthase activity has also been shown to decline after more than four hours of fat infusion (Boden, Chen, Ruiz, White, & Rossetti, 1994). However, short-term effects of lipid oxidation interference with glucose utilisation might not explain the effects of hyperlipidaemia. The effects are slow, implicating protein synthesis, and the mechanism involves insulin-stimulated glucose transport rather than intracellular events downstream from glucose uptake (Boden, 1997).

Roden et al. (1996) investigated the short term and long term effect of FFA on glucose metabolism by estimating the skeletal muscle concentration of glucose-6-phosphate and glycogen. This was achieved using the nuclear magnetic resonance (NMR) technique every fifteen minutes in nine healthy individuals in the presence of low or high free plasma fatty acids under euglycaemic hyperinsulinaemic clamp conditions for six hours. It was found that FFA induce insulin resistance in humans by initial inhibition of glucose transport/phosphorylation, and this subsequently reduces the rate of muscle glycogen synthesis. Dresner et al. (1999) undertook an additional study in fourteen healthy volunteers, using NMR technique, to distinguish whether the defect is in glucose transport or in phosphorylation. The study found that in people with type 2 DM, impairment of insulin-stimulated glucose use by muscle was largely due to reductions in insulin-stimulated glucose transport. The authors also explored the cause of the glucose transport defect due to FFA by performing the muscle biopsy study before and after
hyperinsulinaemic-euglycaemic clamp in seven individuals (Dresner et al., 1999). IRS-1 associated phosphatidyl inositol (PI) 3-kinase activity was measured in muscle biopsy preparation. Following insulin stimulation, IRS-1-associated PI 3-kinase activity, increased fourfold over basal activity in the glycerol infusion studies. In contrast, during the lipid infusion studies, there was a >90% blunting in the insulin-stimulated IRS-1-associated PI 3-kinase activity compared with the glycerol infusion studies, and there was no detectable increase in activity compared with the basal state. It was suggested that the reduced PI 3-kinase activity may be due to a direct effect of intracellular FFA (or some FFA metabolite) on PI 3-kinase and/or secondary to alterations in upstream insulin signalling events. From the NMR and muscle biopsy experimental setups, it was concluded that increased concentrations of plasma FFA induce insulin resistance in humans through inhibition of glucose transport activity, possibly as a consequence of reduced IRS-1-associated PI 3-kinase activity (Dresner et al., 1999).

Shulman (2000) proposed a unifying hypothesis for common forms of human insulin resistance based largely on the above observations of an NMR and muscle biopsy study (Dresner et al., 1999). The author stated that an increased concentration of intracellular fatty acid metabolites, such as diacylglycerol, fatty acyl CoA’s, or ceramides, activates a serine/threonine kinase cascade (possibly initiated by protein kinase C-θ), leading to phosphorylation of serine/threonine sites on IRS. Serine-phosphorylated forms of these proteins fail to associate with or to activate PI 3-kinase, resulting in decreased activation of glucose transport and other downstream events.
2.2.4 Oxidative stress and insulin resistance

Free radicals are extremely reactive molecules with one or more unpaired electrons in the outer valence shell (Halliwell, 2007). Most biological molecules contain paired electrons and are thus less reactive (Halliwell, 1989). Oxidative stress exists when oxidising species exceed antioxidant capability, causing damage to cellular components. It is defined as an imbalance between prooxidant and antioxidant factors in favour of the prooxidants (Sies, 1997). Oxidative stress appears to be involved in many diseases and it could be a cause or consequence (Briehl, 2015). Free radicals are generated in small amounts by normal physiological processes, in larger amounts by the immune system and can also come from exogenous sources (Halliwell, 1991). About 1-3% of the oxygen consumed each day becomes a free radical or reactive oxygen species through mitochondrial metabolism (Carrell, Winterbourn, & Rachmilewitz, 1975). Oxidative stress and low grade inflammation are mostly discussed together due to their interplay in metabolic diseases. The biochemical mediators of inflammation include cytokines, neuropeptides, growth factors and neurotransmitters (Omoigui, 2007). “Low grade inflammation” is a condition where inflammatory markers are elevated in the absence of the classic signs of inflammation (Kushner, Samols, & Magrey, 2010). Our research team believe that inflammation generated from any cause of cellular damage (eg infection, autoimmunity, and trauma) causes oxidative stress which causes further cellular damage and inflammation in a self-perpetuating sequence.

An excessive calorie intake increases the flux of substrate through the citric acid cycle and generates excess mitochondrial NADH and reactive oxygen species (Maddux et al., 2001).
Citrate enters the citric acid cycle and is converted to isocitrate by NAD$^+$-dependent isocitrate dehydrogenase which generates NADH. Excessive NADH cannot be dissipated by oxidative phosphorylation (or other mechanisms) resulting in an increase in the mitochondrial proton gradient. When, a mitochondrial proton gradient increases, single electrons are transferred to oxygen, leading to the formation of free radicals, particularly superoxide anion (Maechler, Jornot, & Wollheim, 1999).

Over-nutrition and a sedentary life style lead to increased glucose and FFA loads in cells, and their transformation in energy is accompanied by an increase in the generation of free radicals (Ceriello & Motz, 2004). The muscle cells and adipocytes protect themselves from this condition by reducing glucose uptake, thereby producing insulin resistance. Glucose and FFA overload in insulin-independent tissues, such as β-cells and endothelium, thereby generating free radicals. The free radicals, in turn, induce a dysfunction of both β-cells and endothelial cells (Ceriello & Motz, 2004). β-cell dysfunction is characterised by a decreased first-phase insulin secretion, which in turn, produces the clinical picture of impaired glucose tolerance. The latter situation is clinically characterised by increased postprandial hyperglycaemia, which induces oxidative stress. The persistence of this condition produces an exhaustion of β-cells, leading to overt diabetes. Oxidative stress produced both during impaired glucose tolerance and overt diabetes may contribute to the development of CVD (Ceriello & Motz, 2004).

Ceriello (2000) hypothesised that hyperglycaemia and insulin resistance can either directly leads to insulin deficiency or indirectly leads to insulin deficiency through oxidative stress (Figure 2.2). Insulin resistance has been significantly correlated with oxidative stress in several studies.
(Dokken, Saengsirisuwan, Kim, Teachey, & Henriksen, 2008; Ogihara et al., 2004; Paolisso & Giugliano, 1996; Sankhla et al., 2012; Shimosawa et al., 2003; Singh, Carey, Watson, Febbraio, & Hawley, 2008).

**Figure 2.2** Conceptual framework of Ceriello (2000): Oxidative stress is a link between insulin resistance and reduced insulin secretion

**2.2.5 Oxidative stress and diabetic complications**

Oxidative stress is associated with DM and its complications (Henriksen, Diamond-Stanic, & Marchionne, 2011; Houstis, Rosen, & Lander, 2006). The unifying hypothesis of oxidative stress leading to DM complications was published in 2001 (Brownlee, 2001). When the electrochemical potential difference generated by the proton gradient across the inner mitochondrial membrane is high, the lifetime of superoxide-generating electron-transport
intermediates, such as ubisemiquinone, is prolonged. There seems to be a threshold value above which superoxide anion production is markedly increased (Korshunov, Skulachev, & Starkov, 1997). Du et al. (2001) found that hyperglycaemia increases the proton gradient above this threshold as a result of overproduction of electron donors by the Krebs cycle. This, in turn, causes a marked increase in superoxide anion production by endothelial cells. As hyperglycaemia-induced overproduction of mitochondrial superoxide causes a decrease in glyceraldehydes-3-phosphate dehydrogenase activity (Du et al., 2000), the effect of hyperglycaemia on polyol pathway flux may reflect the accumulation of glycolytic metabolites, such as glucose, upstream of glyceraldehydes-3-phosphate dehydrogenase activity. Excess superoxide anion partially inhibits the glycolytic enzyme glyceraldehydes-3-phosphate dehydrogenase, thereby diverting upstream metabolites from glycolysis into pathways of glucose over-utilisation. This results in increased flux of dihydroxyacetone phosphate to diacylglycerol, an activator of protein kinase C, and of triose phosphates to methylglyoxal, the main intracellular advanced glycation endproduct precursor. Increased flux of fructose-6 phosphate to UDP-N-acetylglucosamine increases modification of proteins by O-linked N-acetylglucosamine (GlcNAc), and increased glucose flux through the polyol pathway consumes NADPH and depletes GSH (Brownlee, 2001) (Figure 2.3).
Figure 2.3 Flowchart showing a potential mechanism by which hyperglycaemia-induced mitochondrial superoxide overproduction activates pathways of hyperglycaemic damage (Brownlee, 2001).

Section summary: Multiple biochemical, metabolic, and signal transduction pathways contribute to insulin resistance. Obesity and its co-morbidities are associated with a state of oxidative stress and inflammation. Moreover, oxidative stress has been established as a biochemical factor associated with type 2 DM complications. Oxidative stress and adipocytokines could lead to insulin resistance and ultimately MetS. The effects of oxidative stress, chronic inflammation and dyslipidaemia in the blood flow properties in MetS remain unclear, and thus, will be investigated in this study. The possible effects of components of MetS, such as obesity, dyslipidaemia, DM and hypertension, on haemorheology are discussed in the following section.
2.3 Concept of haemorheology and its association with components of MetS

2.3.1 Haemorheology

Copley (1952) first introduced the term ‘haemorheology’, and defined it as being ‘concerned with the deformation and flow properties of cellular and plasmatic components of blood in macroscopic, microscopic and submicroscopic dimensions, and with the rheological properties of the vessel structure which directly comes in contact with the blood.’ Later, Copley and Seaman (1981) extended the scope of the definition and added, ‘Haemorheology is also the study of the interaction of blood or its components and the vascular system with added foreign materials, such as drugs, plasma expanders, or prosthetic devices. Thus, haemorheology is the study of how the blood and blood vessels can function and interact as parts of the living organism.’ Clinical haemorheological studies deal with pathological haemorheological abnormalities and aim to evaluate their links to diseases, clinical diagnostic as well as therapeutic approaches (Stoltz, Singh, & Riha, 1999b).

2.3.2 Haemorheological parameters

The present study measured erythrocyte deformability, erythrocyte aggregation, erythrocyte morphology and whole blood viscosity (WBV) as haemorheological variables.

Erythrocyte deformability

Erythrocytes need to change their shape in order to pass through the capillary bed and oppose the flow resistance and this is directly related to their deforming capacity (Chien, 1987). Erythrocyte deformability reflects the ability of the erythrocytes to change its shape under shear stress while flowing through a large as well as small vessel in the cardiovascular system (Stoltz et al.,
The erythrocytes demonstrate pronounced resilience during deformation but revert back to their original shape once the shear stress is removed (Marchesi, 1983). The erythrocyte membrane composition and its cytoplasmic contents are the two main factors that determine erythrocyte deformability (Stoltz et al., 1999a). The mean cell volume of the erythrocyte is approximately 90 fl. 97 µm$^2$ of surface area is required to enclose a sphere with a volume of 90 fl. However, the mean surface area of the erythrocyte is 140 µm$^2$ (Chien, 1987). This excess surface area allows the erythrocyte to deform. Decreased deformability will result in impaired perfusion and oxygen delivery in peripheral tissues (Yedgar et al., 2002).

**Erythrocyte aggregation**

Aggregation is the collection of individual erythrocytes to form a collection of doublets, triplets and so on. Two important parameters that determine the aggregation are the average size of rouleaux (aggregate size) and the rate at which erythrocytes adhere (Bertoluzzo, Bollini, Rasia, & Raynal, 1999).

Two mutually exclusive models of erythrocyte aggregation have been accepted: ‘Bridging model’ and ‘Depletion model’ (Neu & Meiselman, 2002). In the bridging model, it is proposed that, erythrocytes aggregate when disaggregation forces of the erythrocytes (electrostatic repulsion, membrane strain and mechanical shearing) are exceeded by the bridging forces due to adsorption of macromolecules onto adjacent cell surfaces (Chien & Jan, 1973; Chien & Sung, 1987). Increase in plasma adhesion proteins, particularly fibrinogen has been considered as a major bridging plasma protein (Letcher, Chien, Pickering, Sealey, & Laragh, 1981). The
fibrinogen specific binding to the erythrocyte membrane surface has also been reported (Lominadze & Dean, 2002). Reduction in the concentration of fibrinogen has been reported to improve the haemorheological abnormalities (Schuff-Werner et al., 1989). Fibrinogen enhances the formation of robust clusters of erythrocytes even in microcapillary flows (Brust et al., 2014). The depletion model proposes that, aggregation occurs due to lower localised protein or polymer concentration near the cell surface as compared with the suspending medium. The exclusion of the macromolecules near the cell surface leads to the osmotic gradient between the intercellular polymer-poor depletion zone and the bulk phase, resulting in solvent displacement into the bulk phase, ultimately causing depletion interaction (Bäumler et al., 1996). There are also certain cell specific factors (intrinsic properties of the erythrocytes) that promote erythrocyte aggregation, which is specifically termed as ‘erythrocyte aggregability’ (Rampling, Meiselman, Neu, & Baskurt, 2004).

**Erythrocyte morphology**

The study of erythrocyte morphology is of great importance in haemorheology, since the deformability of the circulating cells has a fundamental influence on the rheological properties of the blood (Dintenfass, 1971). Erythrocytes may respond by changing their morphology to any form of insult in their membrane or biochemical composition. Treatment of physiological biconcave discoid erythrocytes with various agents can transform them into their two extreme opposite forms: stomatocytes and echinocytes (Deuticke, 1968). Amphipathic compounds that intercalate themselves into the cytoplasmic half of the lipid bilayer (e.g. cationic amphipaths such as phenothiazine tranquilizers) cause expansion of the inner half relative to the outer half,
causing invagination and thus eventually leading to stomatocyte formation. However, compounds that are preferentially distributed into the outer half (e.g., anionic amphipaths such as free fatty acids) expand the outer half relative to the inner half and induce crenations leading to echinocyte formation (Deuticke, 1968; Sheetz & Singer, 1974).

**Whole blood viscosity (WBV)**

Blood viscosity refers to the thickness of the blood. It is the intrinsic resistance to the blood flow developed due to the frictional force between adjacent layers of flowing blood. During flow, layers of blood move parallel to one another at different rates which creates a velocity gradient between layers. This velocity gradient is known as the ‘shear rate’ and it is measured in reciprocal seconds, $s^{-1}$). The force required to produce this velocity gradient is defined as the ‘shear stress’ and is measured in newtons per square metre ($Nm^{-2}$). Hence, in the concept of physics, viscosity is defined as the ratio of shear stress to shear rate (Stuart & Kenny, 1980). Unlike in water and plasma, a linear relationship does not exist between shear rate and shear stress in blood (non-Newtonian fluid), and therefore, viscosity of the blood does not remain constant at different shear stresses applied. The viscosity of the blood increases exponentially at low shear rates (Stuart & Kenny, 1980). This increase is attributed to the larger molecular weight plasma proteins (fibrinogen and certain globulins). Fibrinogen overcomes the zeta potential (repulsive electric potential) between erythrocytes and easily could form rouleaux; which increases the WBV.

In a blood vessel with diameter of less than 500 µm, both the haematocrit and WBV are reduced. This is known as Fahraeus-Lindqvist effect (Fahraeus & Lindqvist, 1931). This effect has been
attributed to the migration of erythrocytes to the centre of the blood vessel, thereby creating a cell-poor plasma layer on the wall and a cell-rich central core (Skalak, Chen, & Chien, 1972). The diameter of this cell poor layer increases as the diameter of the blood vessel decreases (Gupta & Seshadri, 1976). This axial drifts of the erythrocytes lowers the resistance to flow (Palmer, 1965). WBV also depends on vascular geometry and plasma constituents, and all of the abnormalities that cluster in MetS (Expert Panel on Detection Evaluation Treatment of High Blood Cholesterol in Adults, 2001) are likely to impair the above mentioned factors, and thus affect WBV independently.

The haemorheological factors are found to be altered in all of the components of MetS. The next sections review the various studies that demonstrate alterations of rheology in individual MetS components: DM, hypertension, dyslipidaemia, obesity, oxidative stress and chronic inflammation.

2.3.3 Altered haemorheology and diabetes mellitus

Altered haemorheology plays an important role in the pathophysiology of blood circulation (Soutani, Suzuki, Tateishi, & Maeda, 1995) and in the complications associated with DM (Little, 1976; Satoh, Imaizumi, Bessho, & Shiga, 1984; Zimmermann et al., 1996). Increased erythrocyte aggregation has been positively correlated with the complications of DM (Demiroğlu, Gürlek, & Barişta, 1999). The patients with Type 2 diabetes with clinically evident late complications have elevated erythrocyte aggregation regardless of the degree of metabolic control (Demiroğlu et al., 1999). It has been shown that an increased degree of erythrocyte
aggregation is related to the progression of neuropathy in DM patients (Husstedt, Grotemeyer, Evers, Staschewski, & Wertelewski, 1997).

Erythrocyte aggregation was found to be higher in the group of patients with impaired glucose tolerance after the second and third hour of glucose intake, but not immediately after the first hour when compared to healthy controls (Koltai et al., 2006). WBV was higher only after the first hour of glucose intake. By contrast, there was no significant difference noted in erythrocyte filterability at any point of time after glucose intake between healthy controls and patients with impaired glucose tolerance. As there was no change in erythrocyte aggregation after the first hour, the authors concluded that the increased erythrocyte aggregation after glucose consumption could be due to elevated insulin concentration rather than the effect of acute hyperglycaemia (Koltai et al., 2006). However, the findings of this study should be interpreted cautiously. WBV was high at first hour of glucose intake without affecting the other two important haemorheological parameters: erythrocyte aggregation and erythrocyte deformability. Similarly, erythrocyte aggregation was reported to be higher only at the second and third hour of glucose intake without any concomitant changes in the other two rheological profiles at that point of time. Hence, isolated changes of haemorheological profile after glucose intake does not necessarily provide the broad picture of the short term effect of hyperglycaemia. There is possibly a much more complex interaction of metabolic states and biomolecules that produce the clinically significant alterations in haemorheology.

Erythrocyte aggregation has been negatively correlated with insulin sensitivity (Brun, Varlet-Marie, & Raynaud de Mauverger, 2012; Brun, Varlet-Marie, Raynaud de Mauverger, & Mercier, 2012) (Figure 2.4), and it has been argued that increased erythrocyte aggregation could be an
early sign of insulin resistance (Brun, Varlet-Marie, & Raynaud de Mauverger, 2012; Brun, Varlet-Marie, Raynaud de Mauverger, et al., 2012). Koltai et al. (2006) on the other hand, suggested that in vivo glucose toxicity due to hyperglycaemia could be the reason behind elevated WBV, since there was a lack of change in erythrocyte deformability throughout the experiment, and WBV only became elevated after the first hour (mean glucose concentration 12.58 mmol/L) of 75 g glucose intake in the same study. A large community based study, however, has suggested that WBV is associated with insulin resistance and is the independent predictor of type 2 DM (Tamariz et al., 2008). In the study, estimated WBV (shear rate of 208 s$^{-1}$) was found to be positively correlated with higher BMI, waist circumference, waist:hip ratio, systolic blood pressure, fasting glucose, insulin, fibrinogen, white blood cells, and triglyceride levels. By contrast, estimated WBV (shear rate of 208 s$^{-1}$) was inversely associated with HDL-C concentration (Tamariz et al., 2008). DM was positively associated with greater estimated WBV, rising from 11.2 per 1,000 person-years in the lowest quartile of estimated blood viscosity to 20 per 1,000 person-years in the highest quartile. This nearly 2-fold gradient persisted after adjustment for age, sex, and race. Even after simultaneous adjustment for age, sex, race, parental history of diabetes, education, field center, BMI, waist:hip ratio, smoking, and physical activity, the risk for the incident of type 2 DM was strongly associated with elevated estimated WBV (Tamariz et al., 2008).
Figure 2.4 Correlation between erythrocytes aggregability index and insulin sensitivity (Brun, Varlet-Marie, & Raynaud de Mauverger, 2012). The figure shows the decreased erythrocyte aggregability when insulin sensitivity is increased. However, in the figure, erythrocyte aggregation approaches to almost zero at the insulin sensitivity of 150 units, which is not true in-vivo physiologically. Also, at the insulin sensitivity of 80 to 100, there are values falling farther from the regressional line.

Glucose oxidation and protein glycation, caused by diabetes-associated hyperglycaemia and insulin resistance, can induce a number of modifications in the mechanical and rheological properties of erythrocytes. To demonstrate the direct effect of glucose on erythrocyte deformability, erythrocytes from healthy volunteers were incubated in-vitro for a maximum of two hours with increasing concentrations of glucose (Shin, Ku, Suh, & Singh, 2008). The erythrocyte elongation index (EI) given by \((L - W)/(L + W)\), where \(L\) and \(W\) are the major and minor axes of the ellipse, was used as a measure of erythrocyte deformability. For all of the
glucose concentrations and incubation times (minimum 30 minutes), EI of the erythrocytes incubated with glucose was reduced when compared to erythrocytes incubated in glucose-free media. Also, EI decreased as glucose concentration increased (Shin et al., 2008). Similarly, in another study, erythrocyte deformability was correlated with glycated hemoglobin (Babu & Singh, 2004). The membrane protein glycation may in part determine the structural alterations in erythrocytes, its deformability and WBV (Schmid-Schonbein, Wells, & Goldstone, 1969).

Structural alterations to erythrocytes can be viewed microscopically. Studies have shown that the perimeter of the cell increases and the area decreases, along with the development of irregularity in the membrane with increased blood glucose concentration (Babu & Singh, 2004). Spherocytes were reported in type 1 diabetics, whereas both spherocytes and echinocytes were reported in type 2 diabetics in the peripheral blood smear (Cimbaljevic et al., 2007). The authors (Cimbaljevic et al., 2007) stated that the spherocytosis, observed in both types of diabetes, appears to be associated with hyperglycaemia. In comparison, echinocytosis in patients with type 2 DM was possibly related to the abnormal plasma lipid profile and the increased concentration of lipid peroxides.

2.3.4 Altered haemorheology and hypertension

Blood pressure depends on the shear rate of blood flow and peripheral vascular resistance. These two factors, in turn, depend on several rheological factors such as erythrocyte aggregation, deformability, haematocrit, vessel geometry and plasma viscosity (Pirrelli, 1999; Sandhagen, 1999). Specifically, blood pressure is calculated from cardiac output multiplied by peripheral
resistance. Hence, factors that affect these two parameters influence blood pressure (Pirrelli, 1999; Sandhagen, 1999).

The principle factors that regulate the peripheral resistance are as follows (Pirrelli, 1999):

1. vessel radius

   Active regulation by:
   
   A) local factors – metabolites (adenosine, K\(^+\), H\(^+\), high PCO\(_2\), low PO\(_2\), osmolarity);
   
   • autacoids (prostaglandins, thromboxanes, nitric oxide, acetylcholine, etc.)
   
   • myogenic tone
   
   • temperature

   B) neural factors (norepinephrine, acetylcholine, nitric oxide)

   C) humoral factors (cathecolamines, angiotensin II, vasopressin)

   Passive regulation by:

   A) structural factors (plaque formation, vascular compliance, endothelial morphology)

   B) transmural pressure (intravascular – extravascular)

2. vessel length

3. number of vessels

4. vessel geometry

5. erythrocyte rheology (Haemorheology)

6. white blood cell deformability

7. platelets

The mechanical and biochemical characteristics of erythrocytes are altered in hypertension (Meyer & Garay, 1981), and there is a general membranous defect in the essential hypertension
(Naftilan, Dzau, & Loscalzo, 1986). Transportation of ions across the erythrocytic membrane is also altered by hypertension (Postnov et al., 1987; Postnov et al., 1988; Postnov, Orlov, Shevchenko, & Adler, 1977).

It has also been reported that the concentration of free calcium ions is increased in the cytoplasm of cells in essential hypertension (Postnov, 1990). These defects in ion transport and concentration may account for erythrocyte membrane alterations and cytoskeleton defects. Phosphorylation of spectrin is decreased due to increased cytosolic calcium concentrations, and hence, erythrocytes are less deformable (Cicco & Pirrelli, 1999). The ATP pool is affected in patients with essential hypertension (Tripsa & Zaciu, 1990), and this leads to changes in membranous properties and erythrocyte morphology. Teodori et al. (2007) showed that the erythrocytes of healthy patients with familial hypertension were less biconcave, whereas the erythrocytes of essential hypertension patients were more biconcave than those of controls without familial hypertension. They offered no explanation for the finding (Teodori et al., 2007). In fact, the finding suggests that rheological factors are pronounced when multiple abnormal factors, as seen in MetS, cluster together. In the study (Teodori et al., 2007), the hypertensive patients were of new onset and were not on any pharmacological antihypertensive treatment, and exclusion criteria were rigorous in the study. The patients with essential hypertension did not have any other chronic diseases and had normal BMI. Hence, it is quite possible that manifestations of clinically abnormal haemorheological parameters depend on long term metabolic alterations/diseases rather than a short term acute abnormality.

Erythrocyte morphology was found to be further altered in essential hypertensive patients suffering from end-organ damage (Turchetti et al., 1997). The same trend was shown by WBV.
and fibrinogen concentration (i.e. significantly high in essential hypertension and even higher in essential hypertension with end-organ damage) (Turchetti, Bellini, Guerrini, & Forconi, 1999). Hence, it can be surmised that morphological changes depend on the complexity and severity of underlying pathology.

Postnov et al. (1987) revealed a threefold increase in monoconcave cup-shaped cells in essential hypertension patients in comparison to normotensive cases using phase contrast microscopy. When the normotensive erythrocytes were treated with 12-Otetradecanoylphorbol-13-acetate, an activator of protein kinase C, the concentration of monoconcave cup-shaped cells was seven to nine folds higher in comparison to that in untreated normotensive erythrocytes. It was suggested that the appearance of unusual cup-shaped monoconcave cells may be due to increased activity of protein kinase C or by the alteration of the membrane skeleton (Postnov et al., 1987; Postnov et al., 1988).

Treatment of renal anaemic patients with erythropoietin increases blood pressure (Raine & Roger, 1991). Sandhagen (1999) suggested that reductions in erythrocyte fluidity could be an important factor affecting the resultant increase in blood pressure. Pirrelli (1999) reported that altered haemorheology could be one of the numerous causes of hypertension and argued that the chronic increased shear stress and hyperviscosity changes the physical conditions of the vascular wall, and/or the general vascular geometry. Altered haemorheology and increased release of endothelial vasoactive factors induce an increase in peripheral resistance, which can increase arterial hypertension (Pirrelli, 1999). To investigate the possible relationship of erythrocyte aggregation and essential hypertension, an experiment was carried out in hypertensive patients
(Mchedlishvili, Tsinamdzvirishvili, Beritashvili, Gobejishvili, & Ilencko, 1997). The erythrocyte aggregation index was found to be higher in hypertensive patients when compared with controls. Furthermore, the erythrocyte aggregation index was higher in severe hypertensive patients compared with mildly hypertensive patients (Mchedlishvili et al., 1997). Total peripheral resistance in the hypertensive patients was found to be higher when blood rheological disorders were severe (Mchedlishvili et al., 1997). Diastolic blood pressure, total peripheral resistance and erythrocyte aggregability index were reduced following treatment with a calcium antagonist, nifedipin. The authors reported that altered erythrocyte aggregation might be a mechanism (or at least a partial mechanism) for the elevation of peripheral resistance and the resultant rise of diastolic arterial pressure in essential hypertension (Mchedlishvili et al., 1997). The increased erythrocyte aggregation index in severe hypertension patients compared with mild hypertension patients in the same study also suggests that altered rheology is associated with the severity of the hypertensive pathology. Furthermore, the anti-hypertensive drugs, Ca++ channel blocker and angiotensinogen converting enzyme inhibitor, have been shown to improve haemorheology in hypertensive patients (Khder et al., 1998; Muravyov, Zaitsev, Muravyov, Yakusevich, & Sirotkina, 1998).

Plasma volume is decreased due to an increased filtration pressure in hypertension, leading to an increased haemoconcentration, and hence, elevated WBV (Parving & Gyntelberg, 1973; Tarazi, Frohlich, & Dustan, 1968). An increased adrenergic sympathetic nervous system activity may also decrease plasma volume, and hence, lead to an increased WBV (Linde et al., 1993). Letcher and his colleagues (Letcher et al., 1981) measured blood and plasma viscosity in untreated essential hypertension patients and compared them with blood and plasma viscosity in control
patients. Blood viscosity values measured at six different shear rates were significantly correlated with blood pressure, and WBV was higher in hypertensive patients when compared with healthy controls. When the haematocrit-matched hypertensive subgroup was compared with controls, WBV was still higher in patients with essential hypertension. Since the viscosity of defibrinated blood was similar in normal and hypertensive patients with matched haematocrit, higher fibrinogen concentration was suggested as one of the main factors causing the elevated WBV in the hypertensive patients. However, other studies have not found any correlation between fibrinogen and plasma viscosity and erythrocyte aggregation in hypertensive patients (Linde et al., 1993). In the same study, WBV was found to be higher in the hypertensive group even after correction for haematocrit. Therefore, Linde et al. (1993) concluded that some other proteins such as albumin and haemorheological factors (apart from fibrinogen level and haemoconcentration) play a role in increasing WBV in essential hypertension.

2.3.5 Altered haemorheology and dyslipidaemia

Dyslipidaemia is one of the major components of MetS and an important risk factor of CVDs. Most of the lipids in the erythrocyte membrane originate from the plasma lipoproteins, since erythrocytes cannot synthesise lipids (Gottlieb, 1980; Lange & D'Alessandro, 1977). Erythrocyte membrane cholesterol content is high in hypercholesterolaemic patients, and LDL is mainly responsible for increased cholesterol delivery to erythrocytes (Cooper, 1977; Schick & Schick, 1985). Increased delivery of cholesterol changes the erythrocyte membranous properties (Babu, 2009b). To demonstrate the effect of cholesterol on erythrocyte morphology, the shape of erythrocytes from a normal population was compared with that in both normocholesterolaemic
diabetics and hypercholesterolaemic diabetics. The range of blood glucose levels was the same in both normo- and hyper-cholesterolaemic groups. The changes in shape parameters of hyperglycaemic samples with normo- compared with hyper-cholesterol levels were found to be significant. More deviation was found in the erythrocyte shape in diabetic patients with high cholesterol than in those patients with normal cholesterol levels (Babu, 2009b).

Zhao et al. (2006) investigated the effect of triglyceride, another important lipid component, on haemorheology, using lipoprotein lipase-deficient mice. Erythrocyte deformability and electrophoretic mobility were reduced, whereas osmotic fragility was increased in lipoprotein lipase-deficient mice when compared to control mice (Zhao et al., 2006). Erythrocytes of lipoprotein lipase-deficient mice also had irregular protrusions in their surface as revealed from scanning electron microscopy (Zhao et al., 2006). Similarly, in another study, it was demonstrated that, the rate of rouleaux formation was increased in the blood with a high concentration of triglyceride, and the rate of rouleaux formation was significantly correlated with the plasma level of triglyceride (Cicha, Suzuki, Tateishi, & Maeda, 2001; Maeda, Cicha, Tateishi, & Suzuki, 2006).

Dyslipidaemia changes the normal lipid composition of plasma, which ultimately changes the membrane lipid composition of erythrocytes. It has been demonstrated that the incubation of washed erythrocytes with FFA and its derivatives changes the erythrocyte morphology, reduces its fluidity and increases aggregation (Zavodnik, Zaborowski, Niekurzak, & Bryszewska, 1997). Changes in the lipid composition of plasma and the erythrocyte membrane may enhance the
interaction between erythrocytes and plasma proteins, and subsequently lead to hyperaggregation (Cicha et al., 2001; Maeda et al., 2006).

If dyslipidaemia causes the alterations in haemorheology, then a reduction in abnormal lipids from plasma should normalise the altered haemorheology. It has been shown that, after direct absorption of lipoproteins (DALI) apheresis for five consecutive sessions in six hypercholesterolemic patients, erythrocyte aggregation was reduced by 42% and WBV by 10% (Bosch, Wendler, Jaeger, & Samtleben, 2001). Similarly, ten heterozygous familial hypercholesterolaemic patients showed a significant reduction in plasma viscosity and erythrocyte aggregation after undergoing heparin-induced extracorporeal lipoprotein precipitation treatment (Schuff-Werner et al., 1989).

The beneficial effects of lipid lowering therapy by statin drug in haemorheology have also been seen in several studies (Abrashkina, Shaalali, Pakhrova, Shutemova, & Nazarova, 2010; Jay, Rampling, & Betteridge, 1990; Levy, Leibowitz, Aviram, Brook, & Cogan, 1992; Medvedev & Skoriatina, 2012). After three months of treatment with lovastatin, fourteen patients with familial hypercholesterolaemia showed a reduction in erythrocyte membrane cholesterol content and an increase in phospholipids content. There was also a significant improvement in the erythrocyte deformability and a significant reduction in erythrocyte aggregation (Martínez, Vayá, Gil, et al., 1998). Significant positive effects on blood haemorheology mediated by the efficient reduction of cholesterol as described in the above studies indicate that dyslipidaemia could be one of the prime causes of increased erythrocyte aggregation in MetS.
Increased erythrocyte aggregation was found to be largely related to dyslipidaemia among different components of MetS (Aloulou, Varlet-Marie, Mercier, & Brun, 2006). Ninety patients were classified into four groups according to the number of positive components of MetS (NCEP ATP III scoring) present, to study the change in haemorheology across different groups. No significant changes in erythrocyte rheological properties across classes of NCEP ATP III score were found. This is probably due to a small number of participants across the group. There were only seven participants with three positive MetS components and eleven with four positive components. None of the participants were positive for all of the five components. Moreover, mean value of BMI, waist circumference, and duration and complications of the diseases (type 2 DM, hypertension) in each group were not shown in the study. The smaller number of sample size in ATP score III and IV; and the different nature and duration of the complications of the diseases could be a reason for the non-significant correlation between rheological parameters and NCEP ATP III score. Erythrocyte aggregation correlated positively with total cholesterol level and negatively with HDL-C level (Aloulou et al., 2006). Erythrocyte aggregation increased in patients with primary hyperlipoproteinaemia (Muller, Ziegler, Donner, Drouin, & Stoltz, 1990; Vayá, Martinez, Carmena, & Aznar, 1993). Erythrocyte aggregation was higher in hyperglycaemic patients when compared to normal controls, and it was even higher in hypercholesterolaemic hyperglycaemic patients when compared to hyperglycaemic patients (Babu, 2009a). Also, a modest increase in cholesterol concentration among polygenic hypercholesterolaemic children has been shown to be associated with increased erythrocyte aggregation (Vayá, Martínez, Dalmau, & Aznar, 1998).
Ultrasound duplex scans showed increased in-vivo erythrocyte aggregation in the veins and arteries of hyperlipidaemic patients compared with those of normolipidaemic patients (Cloutier et al., 1997). Sixty normotensive, normal weight hypercholesterolaemic men with normal triglyceride levels showed a higher aggregation index and disaggregation shear rate threshold when compared to sixteen normocholesterolaemic controls. Erythrocyte aggregation was inversely correlated with HDL-C, HDL_{2-C}, and LpA-I in hypercholesterolaemic patients. In a multiple regression model, HDL_{2-C}, Lipoprotein A-I, and Lipoprotein A-I/A-II emerged as significant factors influencing erythrocyte aggregation index and disaggregation shear rate threshold (Razavian et al., 1994). HDL-C has also been inversely correlated with erythrocyte aggregation in normal participants as well as in coronary heart disease patients (Ruhensroth-Bauer, Mossmer, Ottl, Koenig-Erich, & Heinemann, 1987). One-hundred-and-twelve healthy volunteers were studied to find the effect of lipids on erythrocyte rheological properties (Contreras et al., 2004). Erythrocyte aggregation was significantly positively correlated with total cholesterol, triglyceride, LDL-C and apo B, and significantly negatively correlated with HDL-C and apo A1 (Contreras et al., 2004). It has been demonstrated that erythrocyte permeability in vivo is impaired by high levels of total cholesterol and LDL-C (Lee, Kim, Park, Song, & Lee, 2004). HDL inhibits Ca^{2+}-induced pro-coagulant activity on erythrocyte membranes (Epand et al., 1994). It also competes with LDL for binding with the erythrocyte membrane, competitively inhibiting the LDL-C-induced erythrocyte aggregation, and subsequently reducing WBV (Sloop & Garber, 1997). HDL-C also plays a key role in protecting erythrocyte membranes against oxidative damage (Ferretti, Bacchetti, Busni, Rabini, & Curatola, 2004). Thus, HDL-C may counteract the unfavourable effects of macromolecules such as fibrinogen to reduce erythrocyte aggregation (Razavian et al., 1994).
2.3.6 Altered haemorheology and obesity

Meiselman (1999) questioned whether altered haemorheology was the cause or effect of hypertension and acknowledged that the chicken versus egg problem has not been resolved. Bogar (2002) replied to the question and argued that haemorheology and hypertension are not ‘chicken or egg’, but are two chickens from similar eggs. The egg he referred to was obesity, and he went on to claim that being overweight, and a sedentary lifestyle are the major causes behind abnormal haemorheology and other metabolic interrelated diseases (Bogar, 2002).

Abnormal haemorheological parameters are reported in obesity. Elevated high shear rate WBV (200 s⁻¹, 30 s⁻¹ and 5 s⁻¹) was reported in obese children in comparison to age- and height-matched non-obese children, but there was no difference in low shear rate WBV (1 s⁻¹) and erythrocyte aggregation index (Zhu, Li, Huang, & Neubauer, 2005). The same study also showed that WBV measured at high shear rate was the strong predictor of endothelial dysfunction (Zhu et al., 2005). In addition, increased WBV was shown in 90 obese participants with established CVD at both low and high shear rate in comparison to age- and sex-matched controls, and WBV at both shear rates was correlated with BMI (Rillaerts, van Gaal, Xiang, Vansant, & De Leeuw, 1989).

To study the effect of weight loss on haemorheological parameters, Solá et al. (2004) recruited forty-one patients with severe or morbid obesity, but no other CVD risk factors. Obese patients showed a higher degree of erythrocyte aggregation when compared with forty-five healthy controls (Solá et al., 2004). These obese patients were kept on a very low calorie diet for one month (Sola et al., 2004). Significant reductions were reported in levels of glucose, total
cholesterol, LDL-C, triglyceride, apoB and erythrocyte aggregation together with a concomitant decrease in BMI after the one-month duration. In comparison, no significant changes in fibrinogen, WBV or plasma viscosity were reported (Sola et al., 2004). A significant correlation of BMI with plasma viscosity and erythrocyte aggregation has been demonstrated (Brun, Varlet-Marie, Raynaud de Mauverger, & Mercier, 2011). A decrease in erythrocyte aggregation and improvement in haemorheology after weight loss has been reported in several studies (Fanari et al., 1993; Hankey, Rumley, Lowe, Woodward, & Lean, 1997; Poggi et al., 1994). Improvements in rheology after weight loss in obese patients could be due to complex biochemical and endocrine changes after weight loss. In a study involving sixty-seven severely obese patients (BMI ≥ 35), erythrocyte aggregation decreased after weight loss, and it was suggested that increased erythrocyte aggregation in obesity is the result of increased insulin resistance rather than elevated fibrinogen or dyslipidaemia (Solá et al., 2007).

Elevated haematocrit and tendency of erythrocytes to aggregate in obese participants could be considered to be major factors causing increased WBV. Native WBV and haematocrit were reported to be higher in obese males in comparison to in obese females, but there was no difference in haematocrit-corrected WBV level between the two groups (Maciej et al., 2010). Erythrocyte aggregation index was positively correlated with WBV in both groups. Erythrocyte fluidity was found to be decreased in morbidly obese women (Levy, Elias, Cogan, & Yeshurun, 1993). Altered erythrocyte deformability and aggregation found in obesity (Levy et al., 1993; Maciej et al., 2010; Wiewiora, Sosada, Wylezol, Slowinska, & Zurawinski, 2007) could have an adverse influence in WBV. WBV at high shear rate is influenced by erythrocyte deformability (Schmid-Schonbein et al., 1969). In a study of 109 Japanese obese patients (BMI ≥ 25), it was
shown that blood passage time (BPT) (studied by Micro Channel Array Flow Analyser) was significantly higher in MetS groups than in non-MetS obese groups. BPT was correlated with the severity of MetS (assessed by the number of positive components), as well as with MetS variables such as increases in body weight, BMI, waist circumference, systolic blood pressure, diastolic blood pressure and triglyceride (Satoh et al., 2009). The authors also investigated effects of weight reduction on BPT and found that BPT significantly decreased in patients with a weight reduction of greater than three percentages, but not in patients with a weight reduction of less than three percentages. The decrease of BPT by weight reduction was significantly related to a reduction of waist circumference, systolic blood pressure and pulse wave velocity. These results suggest that various components of MetS and particularly central obesity contribute to decreased erythrocyte deformability (Satoh et al., 2009).

**Section summary:** The study of haemorheology is of great importance in the field of vascular diseases as the deformability and aggregation of the circulating cells has a fundamental influence on the microcirculation properties of the blood. DM, hypertension, dyslipidaemia and obesity (particularly central obesity) are the major components of MetS, and this section focused on the changes in erythrocyte aggregation, WBV, erythrocyte deformability and morphology in different components of MetS. If altered haemorheology is associated with MetS, than its potential usefulness as a bioindicator in the assessment of MetS severity and progression needs to be clarified. Also, the studies that have analysed haemorheology in MetS patients have not associated the alterations with the novel components of MetS such as oxidative stress and chronic inflammation. The next section deals with the possible effect of oxidative stress and chronic inflammation in haemorheology.
2.4 Association of haemorheology with chronic inflammation and oxidative stress

2.4.1 Altered haemorheology and oxidative stress

The changes in blood rheology in MetS can be explained on the grounds of the prooxidant-antioxidant imbalance, because oxidative stress is a common state in metabolic chronic diseases (Baynes, 1991). The erythrocyte is exposed to free radicals from both within the cell and its external environment. It is estimated that between 1 and 3% of the oxygen consumed by aerobic cells becomes a reactive oxygen species (Chance, Sies, & Boveris, 1979). Erythrocyte antioxidant defences are important in maintaining haemoglobin iron in the reduced state and for protecting the plasma membrane of the cell from lipid peroxidation. Erythrocytes possess multiple enzymatic and non-enzymatic antioxidant defense mechanisms to prevent oxidative damages; however, these mechanisms become exhausted during oxidative stress (Richards, Roberts, Mathers, Dunstan, & Butt, 1997).

Certain phenomena, such as oxidation of sulphydryl groups on the membrane of cytoskeleton proteins, oxidation of membrane fatty acid residues or oxidation of the hemoglobin molecule, can alter the membrane properties and shape of the cell (Richards, Roberts, McGregor, et al., 1998). Watanabe et al. (1990), showed that erythrocytes from healthy donors incubated in a hypoxanthine/xanthine oxidase system and hydrogen peroxide had reduced membrane fluidity. Superoxide anion generated outside the membrane (utilising xanthine oxidase-hypoxanthine system) damages only the lipids components of the membranes whereas superoxide anion generated inside the erythrocyte membrane (utilising phenazine methosulphate) (Nishikimi, Rao, & Yagi, 1972) damages the inner protein cross-linking of the membrane and thereby have more severe effect on rheological characteristics (Baskurt, Temiz, & Meiselman, 1998). However,
transport of superoxide anion across erythrocyte membrane has been shown to occur only to be disposed of by reducing substances within the red cell (Richards, Roberts, McGregor, et al., 1998). Also, significant morphologic changes were reported when erythrocytes were incubated with hydrogen peroxide. ‘Spiculated form’ and spheroechinocytes were seen after 5 and 30 minutes of incubation, respectively. Methaemoglobin content was reported to be 96 to 100% in erythrocytes incubated with 2.5 mM peroxynitrite (reactive nitrogen species) for ten minutes in comparison to only 4 to 5% methaemoglobin content in erythrocytes untreated with peroxynitrite. Peroxynitrite treated cells demonstrated changes in the mechanical and physical properties of their membranes when studied by atomic force microscopy (Starodubtseva, Kuznetsova, & Cherenkevich, 2007). Crenated forms (cells with protrusions) of erythrocytes were observed in a dose-dependent manner when blood samples from healthy donors were incubated with peroxynitrite (Starodubtseva, Tattersall, Kuznetsova, Yegorenkov, & Ellory, 2008). The authors reported that the crenation of erythrocytes appears to be caused by both water and ion imbalance between the cells and surrounding medium and cytoskeletal structure changes.

An unfavorable shape change of erythrocytes has also been correlated with increased intraerythrocytic calcium (Turchetti et al., 1998). Watanabe et al. (1990) suggested that membrane disorganisation by free radicals leads to the passive accumulation of calcium inside erythrocytes. It has also been suggested that an increase in insulin concentration impairs the erythrocytic calcium-ATPase activity, leading to increased intraerythrocytic calcium concentration (Grunfeld, Gimenez, Romo, Rabinovich, & Simsolo, 1995). Accumulation of calcium inside the cell further amplifies the damage of free radicals to membranes (Watanabe et al., 1990). Intraerythrocytic ionised calcium and erythrocyte aggregation half-time were
measured in eighteen mild hypertensive patients and compared to fourteen healthy controls (Cicco, Carbonara, Stingi, & Pirrelli, 2001). A significant increase ($P < 0.01$) in the intraerythrocytic ionised calcium was seen when compared to that in the control patients. In comparison, erythrocyte aggregation half time and erythrocyte deformability were reduced, and their reductions were significantly correlated with ionised calcium concentration (Cicco et al., 2001). According to Watanabe et al. (1990), calcium concentration decreases with erythrocyte deformability (Watanabe et al., 1990).

Spectrin, a major protein component of the erythrocyte cytoskeletal structure, performs a variety of membrane functions, including the regulation of membrane deformability and stability (Mohandas, Chasis, & Shohet, 1983). Membrane proteins of erythrocytes, such as spectrin, ankyrin, and protein 4.2, are heavily glycosylated due to oxidation. Oxidative modification of spectrin due to its glycosylation has been attributed to decreased erythrocyte deformability (Madsen et al., 1991). The change in erythrocyte deformability is related to membrane protein peroxidation (Snyder, Fortier, Trainor, Jacobs, & Lob, 1985). Spectrin-haemoglobin complex was seen in the SDS-PAGE electrophoresis when erythrocytes were incubated with an hydrogen peroxide concentration of 135 μM. Subsequently, a dose-dependent increase in spectrin-haemoglobin complex was seen as hydrogen peroxide concentration was increased from 135 to 810 μM. Also, deformability measurements on intact erythrocytes showed that the treatment with hydrogen peroxide resulted in a dose-dependent decrease in whole-cell deformability. Hence, oxidative stress developed in the state of MetS is responsible for abnormal morphology, and reduced deformability of erythrocytes, and a consequential increase in WBV (Schmid-Schonbein et al., 1969).
To demonstrate the effect of an oxidant on erythrocyte deformability and WBV, a high-pressure oxygen sealed chamber was used as an optimal environment for the generation of free radicals (Chen, Chien, Liang, Liu, & Hu, 2011). Two mL of blood from ten healthy male patients with a 45% haematocrit was incubated in the oxygen-sealed high-pressure chamber. Superoxide anion level in the blood increased with increasing oxygen partial pressure (1-2.5 atm) and incubation time (4-16 hours). Generation of superoxide proceeded concomitantly with the formation of lipid peroxides, reduced erythrocytes deformability and elevated WBV. When free radical scavengers catechins were incubated along with blood in the chamber, haemorheological abnormalities were ameliorated (Chen et al., 2011). Similarly, three months oral administration of antioxidant (α-tocopherol nicotinate) improved the haemorheology (increased erythrocyte deformability and decreased WBV) of thirteen type 2 diabetic patients with retinopathy but without any CVDs (Chung, Hau Yu, & Liu, 1998). The authors suggested that an improvement in the erythrocyte membranous properties by tocopherol treatment was the main factor responsible for the improvement of WBV. Reductions in malondialdehyde and WBV without a reduction in plasma viscosity strongly indicate that oxidative stress has an unfavourable effect on WBV. The antioxidant enzymes, SOD and catalase, were shown to protect erythrocyte membrane alterations against activated granulocytes (Baskurt, 1996). Incubation of healthy erythrocytes with oxidant diamide and ferrous sulphate/ascorbate reduced erythrocyte deformability and increased erythrocyte aggregation (Cicha, Suzuki, Tateishi, & Maeda, 1999). These alterations in haemorheology were reduced by the thiol-containing antioxidants dithiothreitol and N-acetylcysteine (Cicha, Tateishi, Suzuki, & Maeda, 1999). Antioxidants also decreased
erythrocyte aggregation induced by photodynamic treatment (Ben-Hur, Barshtein, Chen, & Yedgar, 1997).

In an animal model, it was shown that the concentration of malondialdehyde was higher among rats fed a high cholesterol diet for eight weeks than in those fed a control diet over the same period (Jia et al., 2012). In a group of rats that were fed a high cholesterol diet but also subjected to aerobic exercise, malondialdehyde concentration was close to that in the group of rats not fed with a high cholesterol diet (i.e. the control group). Erythrocyte deformability and electrophoretic mobility (and hence, surface charge) were reduced in the rats fed with the high cholesterol diet when compared to both the control rats and the rats fed with the high cholesterol diet but also subjected to exercise. There were no significant differences among the latter two groups (Jia et al., 2012). The study suggests that hypercholesterolaemia can possibly deteriorate the erythrocyte rheological properties through oxidative stress. Similarly, in the different experiment, it was demonstrated that, erythrocyte aggregation in the microvessels of less than 30 µm was increased in the rabbits when they were fed with cholesterol rich diet in comparison to control rabbits ($P<0.0001$). Erythrocyte aggregation in the rabbits fed with butylated hydroxyl toluene along with cholesterol was significantly less in comparison to that in the rabbits fed with cholesterol alone ($P<0.0001$). In the same way, Vitamin C is also able to prevent cholesterol-induced microcirculatory changes in the rabbit (Freyschuss et al., 1997).

The free radicals generated at different site (external or internal to the erythrocyte membrane) have different patterns of effect on haemorheology (Hebbel, Leung, & Mohandas, 1990). Erythrocyte aggregation was increased, and deformability was reduced by the effect of
superoxide anion generated outside the erythrocytes membrane. When superoxide anion was generated inside the erythrocyte membrane by the phenazine methosulfate, no significant increase in erythrocyte aggregation was noticed despite the reduced deformability (Baskurt et al., 1998).

2.4.2 Altered haemorheology and chronic inflammation

Chronic low-grade inflammation has been hypothesised to play a role in the development of MetS (Dandona et al., 2005). The American Heart Association has indicated that hsCRP measurements might provide information for risk assessment for coronary heart disease beyond that obtained from the established risk factors (Pearson et al., 2003). hsCRP has also been suggested as a marker for the primary prevention of CVD (Ridker, Buring, Cook, & Rifai, 2003). CRP was shown to be high in morbidly obese cases, and multivariate regression analysis showed that CRP predicted erythrocyte aggregability (Vayá et al., 2011). Berliner et al. (2005); demonstrated that erythrocyte aggregation can be used as a biomarker for the detection of low-grade systemic inflammation. Through the use of a receiver operating characteristics curve, the authors showed that erythrocyte aggregation is superior to other commonly used markers, such as fibrinogen and hsCRP, to demonstrate the ongoing inflammation (Berliner et al., 2005). In a group of 234 individuals with and without atherothrombotic risk factors, it was shown that fibrinogen contributed strongly towards erythrocyte aggregation (Assayag et al., 2005). Thirty obese patients without any underlying inflammatory or malignant conditions and thirty-five non-obese healthy volunteers were analysed for erythrocyte aggregation and markers of inflammation (Samocha-Bonet et al., 2003). Significant differences were noted for markers of inflammation
and for the degree of erythrocyte aggregation between the two groups. There was also a significant correlation of BMI with the degree of erythrocyte aggregation, erythrocyte sedimentation rate, fibrinogen, hs-CRP, and leukocyte count. The authors concluded that increased erythrocyte aggregation in obesity could be due to the inflammation present in obesity (Samocha-Bonet et al., 2003).

Adipokines (Kershaw & Flier, 2004; Kim & Moustaid-Moussa, 2000), such as IL-6 (Fried, Bunkin, & Greenberg, 1998), TNF-α (Hotamisligil, Shargill, & Spiegelman, 1993), plasminogen activator inhibitor-1 (Shimomura et al., 1996), and proteins of the rennin-angiotensin system (Cassis, 1994) secreted from adipose tissue, all may significantly alter the haemorheology through complex metabolic, endocrine and genetic interactions. An improvement in haemorheology after weight loss could be related to these metabolic rearrangements (Fanari et al., 1993; Hankey et al., 1997; Poggi et al., 1994; Sola et al., 2004). On the contrary, other authors have reported no significant haemorheological improvement after weight loss (Craveri, Tornaghi, Paganardi, Ranieri, Cietto, et al., 1990; Craveri, Tornaghi, Paganardi, Ranieri, & Giavardi, 1990; Rillaerts, Vansant, Van Gaal, & De Leuuw, 1989). This is probably due to the persistent chronic inflammation that is not necessarily normalised immediately after weight loss. The summary of the haemorheological change in different disease state is shown in the tables below (tables 2.2, 2.3 and 2.4).
Table 2.2: Change in erythrocyte deformability in different components of MetS

<table>
<thead>
<tr>
<th>Authors</th>
<th>Participants</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simmonds et al. (2012)</td>
<td>Type 2 DM female participants before and after exercise</td>
<td>Significant increases in erythrocyte deformability after 12 weeks of exercise.</td>
</tr>
<tr>
<td>Michalska-Małecka and Słowińska-ŁOyńska (2012)</td>
<td>Participants with primary open angle glaucoma with and without arterial hypertensive diseases and controls</td>
<td>Erythrocyte deformability was less in participants with Glaucoma when compared to controls. The difference was further high when controls were compared to glaucoma participants with arterial hypertensive diseases.</td>
</tr>
<tr>
<td>Kowalczyk et al. (2012)</td>
<td>Participants with MetS and Controls</td>
<td>the increase in membrane lipids fluidity at the depth of 5th and 12th carbon atom of fatty acid hydrocarbon chain and significantly decreased fluidity at the depth of 16th carbon atom</td>
</tr>
<tr>
<td>Vayá et al. (2011)</td>
<td>Morbidly obese participants and healthy controls</td>
<td>Erythrocyte deformability was higher in control group.</td>
</tr>
<tr>
<td>Vaya et al. (2011)</td>
<td>Participants with and without MetS</td>
<td>Erythrocyte deformability was higher in participants without MetS.</td>
</tr>
<tr>
<td>Chen et al. (2011)</td>
<td>In vitro, before and after antioxidant treatment</td>
<td>Erythrocyte deformability increased after antioxidant treatment</td>
</tr>
<tr>
<td>Satoh et al. (2009)</td>
<td>Participants with and without MetS</td>
<td>Blood passage time was higher in MetS group. Higher the number of MetS components, higher is the blood passage time.</td>
</tr>
<tr>
<td>Babu (2009b)</td>
<td>Type 2 DM participants with high and low blood total cholesterol</td>
<td>Erythrocyte deformability in normocholesterolaemic diabetic participants was higher than hypercholesterolaemic diabetic participants.</td>
</tr>
</tbody>
</table>
Table 2.2: Contd...

| Authors                        | Participants                                                                 | Results                                                                                                                                 |
|--------------------------------|==============================================================================|-----------------------------------------------------------------------------------------------------------------------------------------|
| Shin et al. (2008)             | Erythrocytes from healthy donor incubated in glucose media of different concentrations | Elongation index decreased as the glucose concentration increased.                                                                         |
| Wiewiora et al. (2007)         | Obese participants Vs Controls                                                | Elongation index of RBC was significantly lower in the obese patients than in the control group at all measured shear rates.            |
| Sola et al. (Sola, Vaya, Santaolaria, et al., 2007) | Obese participants without other cardiovascular risk factors Vs controls | No significant differences were obtained in the EI between obese patients and the control group at any of the shear stresses tested. Obese Participants with MetS had significant lower EI than Obese without MetS. |
| Shin et al. (2007)             | Participants with DM, DM and other complications and controls                 | EI significantly lowered in diabetics when compared to controls. Diabetics without other complications have higher deformability than diabetics with complications. |
| Koltai et al. (2006)           | Normal glucose tolerance group Vs impaired glucose tolerance group            | No significant differences in erythrocyte filtration                                                                                     |
| Szapary et al. (2004)          | Participants with transient ischemic attack or chronic phase of ischemic attack | Erythrocyte filterability was significantly reduced in participants with cerebrovascular diseases.                                           |
| Babu and Singh (2004)          | Participants without CVD complications                                        | Passage time of erythrocytes increases with increasing glucose level                                                                      |
Table 2.2: contd....

<table>
<thead>
<tr>
<th>Authors</th>
<th>Participants</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zita et al. (Zita, Laszlo, &amp; Sandor, 2003)</td>
<td>Participants under haemodialysis</td>
<td>Erythrocyte relative cell transit time higher in participants undergoing haemodialysis</td>
</tr>
<tr>
<td>Presti et al. (2002)</td>
<td>Participants with and without MetS</td>
<td>Whole-blood filterability significantly reduced in MetS participants</td>
</tr>
<tr>
<td>Srour et al. (Srour, Bilto, &amp; Mohidin, 2000)</td>
<td>Before and after exposure of erythrocytes to 10 mM H₂O₂</td>
<td>Erythrocyte deformability was decreased after incubation with 10 mM H₂O₂</td>
</tr>
</tbody>
</table>
Table 2.3: Change in WBV in different components of MetS

<table>
<thead>
<tr>
<th>Authors</th>
<th>Participants</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaya et al. (2011)</td>
<td>Participants with and without MetS</td>
<td>MetS participants have high value</td>
</tr>
<tr>
<td>Irace et al. (2011)</td>
<td>DM Vs Controls</td>
<td>No difference</td>
</tr>
<tr>
<td>Chen et al. (2011)</td>
<td>In vitro, before and after antioxidant treatment</td>
<td>WBV decreased after antioxidant treatment</td>
</tr>
<tr>
<td>Richards and Nwose (2010)</td>
<td>Control Vs DM</td>
<td>WBV high in preDM when compared to Controls</td>
</tr>
<tr>
<td>Maciej et al. (2010)</td>
<td>Obese male Vs Obese female</td>
<td>No difference</td>
</tr>
<tr>
<td>Irace et al. (Irace, Scavelli, Carallo, &amp; Serra, 2009)</td>
<td>Participants with and without MetS</td>
<td>Higher in MetS</td>
</tr>
<tr>
<td>Vekasi et al. (2008)</td>
<td>Diabetic retinopathy participants taking aspirin Vs not taking aspirin</td>
<td>WBV was lower in participants with diabetic retinopathy taking aspirin</td>
</tr>
<tr>
<td>Sola, Vaya, Simo, et al. (2007)</td>
<td>Healthy Vs Obese participants</td>
<td>WBV higher in obese participants</td>
</tr>
<tr>
<td>Kearney-Schwartz et al. (2007)</td>
<td>Healthy controls Vs Hypertensive type 2 DM</td>
<td>WBV higher in Hypertensive Type 2 DM</td>
</tr>
<tr>
<td>Zhang et al. (2006)</td>
<td>1400 office workers</td>
<td>WBV increased with the severity of MetS</td>
</tr>
<tr>
<td>Velcheva et al. (2006)</td>
<td>Participants with ischemic CVD, Vs Controls</td>
<td>WBV was increased in participants with CVD or with risk factors of CVD</td>
</tr>
<tr>
<td>Mannini et al. (2006)</td>
<td>Participants diagnosed with AMI or unstable angina (with and without aspirin resistance)</td>
<td>No change in WBV between with and without aspirin resistance group</td>
</tr>
</tbody>
</table>
Table 2.3: contd....

<table>
<thead>
<tr>
<th>Authors</th>
<th>Participants</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Koltai et al. (2006)</td>
<td>Normal glucose tolerance group Vs impaired glucose tolerance group</td>
<td>High WBV in impaired glucose tolerance group after one hour of glucose consumption</td>
</tr>
<tr>
<td>Zhu et al. (2005)</td>
<td>Non-Obese and obese school children</td>
<td>WBV higher on obese group at shear rate of 200/s and 30/s but not at 1/s</td>
</tr>
<tr>
<td>Ciuffetti et al. (2005)</td>
<td>Essential hypertension</td>
<td>An increased WBV at low shear rate is a predictor of cardiovascular events independently from the effect of several traditional risk factors.</td>
</tr>
<tr>
<td>Sola et al. (2004)</td>
<td>Before and after weight reduction</td>
<td>WBV significantly lowered after 3 months very low calorie diet treatment. WBV reading at 3 months was significantly lower than that of 1 month.</td>
</tr>
<tr>
<td>Devehat et al. (2004)</td>
<td>Type 1 DM, type 2 DM and normal controls</td>
<td>No difference in WBV between type 1 DM and controls; for type 2, WBV was higher at low shear rate when compared to controls</td>
</tr>
<tr>
<td>Turczynski et al. (2003)</td>
<td>Type 2 DM (with retinopathy) Vs Controls</td>
<td>WBV high in DM participants</td>
</tr>
<tr>
<td>Caimi et al. (2003)</td>
<td>Control participants Vs participants diagnosed with AMI</td>
<td>WBV high in AMI participants</td>
</tr>
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</table>
Table 2.3: contd....

<table>
<thead>
<tr>
<th>Authors</th>
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<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presti et al. (2002)</td>
<td>Participants with and without MetS</td>
<td>WBV high in MetS participants</td>
</tr>
<tr>
<td>Devereux et al. (2000)</td>
<td>Controls Vs Essential hypertension</td>
<td>WBV higher in essential hypertension</td>
</tr>
<tr>
<td>Stamos and Rosenson (1999)</td>
<td>Apparently healthy adults with normal triglyceride and and total cholesterol level</td>
<td>Low HDL levels are associated with an elevated blood viscosity</td>
</tr>
<tr>
<td>Sloop and Mercante (1998)</td>
<td>Healthy volunteers</td>
<td>WBV positively correlated with LDL-C and negatively with HDL-C</td>
</tr>
<tr>
<td>Chung et al. (1998)</td>
<td>Type 2 DM participants before and after alpha tocopherol nicotinate treatment</td>
<td>WBV was reduced at all shear rate tested after treatment.</td>
</tr>
<tr>
<td>Lowe et al. (1997)</td>
<td>Prospective study (participants who do not developed cardiovascular incident Vs who developed cardiovascular incidents)</td>
<td>WBV was higher in participants who develop cardiovascular events than who do not developed</td>
</tr>
<tr>
<td>Authors</td>
<td>Participants</td>
<td>Results</td>
</tr>
<tr>
<td>---------------------------------</td>
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</tr>
<tr>
<td>Turchetti et al. (1997)</td>
<td>Diabetics with vasculopathy</td>
<td>Decreased bowls; increased discocytes. Other altered forms: echinocytes and knizocytes</td>
</tr>
<tr>
<td>Babu and Singh (2004)</td>
<td>Diabetics</td>
<td>Perimeter of the erythrocytes increased; area decreased</td>
</tr>
<tr>
<td>Cimaljevic et al. (2007)</td>
<td>Diabetics</td>
<td>Spherocytes in type I and spherocytes and echinocytes in type 2 DM</td>
</tr>
<tr>
<td>Starodubtseva et al. (2008)</td>
<td>Diabetics</td>
<td>62% discocytes , “Crest” erythrocytes, spherocytes, planocytes, macrocytes, and microcytes</td>
</tr>
<tr>
<td>Cicco and Pirrelli (1999)</td>
<td>Essential hypertension</td>
<td>Decreased bowls; increased discocytes</td>
</tr>
<tr>
<td>Turchetti et al. (1999)</td>
<td>Essential hypertension</td>
<td>Decreased bowls; increased discocytes</td>
</tr>
<tr>
<td>Zavodnik et al. (1997)</td>
<td>normal erythrocytes treated with Free fatty acids Palmitate</td>
<td>monoconcave forms, shrinked cells and ghosts cells</td>
</tr>
<tr>
<td>Snyder et al. (1985)</td>
<td>normal erythrocytes treated with H₂O₂</td>
<td>Echinocytes</td>
</tr>
<tr>
<td>Zavodnik et al. (1998)</td>
<td>normal erythrocytes treated with tert-butyl hydroperoxide</td>
<td>Echinocytes, spheroechinocytes, echinocytes III, stomatocytes and ghost cells</td>
</tr>
<tr>
<td>Watanabe et al. (1990)</td>
<td>Normal erythrocytes treated with H₂O₂</td>
<td>Spiculated form and spheroechinocytes</td>
</tr>
</tbody>
</table>
Section summary: MetS refers to the state of oxidative stress and systemic inflammation. Pro-oxidant and inflammatory cytokines induce endothelial dysfunction and vascular function. Though erythrocyte aggregation has been associated with inflammation, there is a paucity of data regarding the effect of inflammatory molecules on erythrocyte morphology, deformability and WBV. Oxidative stress and chronic inflammation generation are well established in MetS, and their effect on insulin resistance and vascular functions in the patients is the subject of ongoing research. However, it is still unclear whether oxidative stress and chronic inflammation generated in MetS affect erythrocyte flow properties. The next section deals with the markers that are often used in assessing peripheral vascular functions and blood flow.
2.5 Ankle Brachial Pressure Index (ABPI), Toe Brachial Pressure Index (TBPI) and peripheral vascular disease

The clustering of risk factors in MetS confers an increased risk of vascular diseases (Isomaa et al., 2001; Lakka et al., 2002). The prevalence of CVDs in MetS patients has been reported to range between 18.3% and 23.3% depending on whether the NCEP ATP II, AHA/NHLBI or IDF definition is applied. In a study by Lakka et al. (2002), male participants with MetS had a nearly three-fold increase in cardiovascular related mortality compared to those without MetS. The increased cardiovascular risk may be explained by the individual risk factors of MetS in association with other, not routinely measured aspects of MetS such as impaired fibrinolysis, oxidative stress, and chronic inflammation. This study also aims to determine whether alterations in haemorheology have any effect on vascular disease.

Reduced ABPI is a marker of atherosclerosis and an indicator of increased cardiovascular risk (Zheng et al., 1997). ABPI, defined as the ratio of the systolic blood pressure in the ankle compared with that in the arm, is a simple, non-invasive, and reliable method to assess PAD. A reduction in ABPI would suggest a fall in blood pressure in an artery at the ankle relative to the central blood pressure, relating to a possible stenosis in the arterial circulation between the aorta and the ankle (Al-Qaisi, Nott, King, & Kaddoura, 2009). The predictive value of ABPI in cardiovascular morbidity and mortality is found to be similar to that of traditional Framingham risk factors (Bhasin & Scott, 2007). Recently, ABPI has been shown to correlate with atheroma burden as measured by whole body contrast enhanced magnetic resonance angiography in patients with symptomatic peripheral arterial disease (PAD) (Weir-McCall et al., 2014). Olijhoek et al. (2004) found a decreased ABPI in MetS participants when compared to non-MetS group.
The overall mortality rate of women with an ABPI of 0.9 or less was found to be five times higher than in the women with normal ABPI values. An ABPI of 0.9 or less was shown to be a strong independent predictor of mortality even in those women who had no history of CVD (Vogt, Cauley, Newman, Kuller, & Hulley, 1993). Similarly, a low ABPI (<0.9) was associated with major cardiovascular risk factors in the elderly patients with systolic hypertension. Age and sex-adjusted relative risk of all causes of mortality were 3.8 times higher in participants with an ABPI less than 0.9 when compared to those in participants with normal ABPI values. The authors suggested that low ABPI values could be a prognostic indicator of subsequent mortality in an elderly population with systolic hypertension (Newman, Sutton-Tyrrell, Vogt, & Kuller, 1993). In another study (Newman et al., 1999), it was shown that the crude mortality rate at six years follow up was highest (32.3%) in those participants with prevalent CVD and a low ABPI ($P<0.9$), and lowest in those with neither of these findings (8.7%, $P<0.01$) (Figure 2.5).

Several studies have used ABPI for diagnosing vascular diseases in diabetic populations (Rabia & Khoo, 2007; Tapp et al., 2003). Several drawbacks, however, have been reported for the use of ABPI. The calcification of the peripheral arteries can make the arteries incompressible, and therefore, the ABPI test (relying on measurement of systolic pressure with an occlusive cuff) becomes non-diagnostic or inaccurate due to artefactually-raised occlusion pressures secondary to medial calcification in the arterial wall (Strandness & Bell, 1965). In type 2 DM, calcification and hardening of the arterial wall can result in a pressure cuff as high as 300 mmHg failing to occlude an artery at the ankle. The validity of measuring ABPI in diabetic patients due to lower limb artery calcification has therefore been questioned (Brooks et al., 2001; Mayfield, Reiber, Sanders, Janisse, & Pogach, 1998). Raines and colleagues estimated that in 5-10% of diabetic
population, ankle pressure cannot be measured by using a cuff due to arterial calcification (Raines, Darling, Buth, Brewster, & Austen, 1976). Given the questionable validity of measuring ABPI in patients with long-standing diabetes, who have already developed calcification of the artery, there is a need for a non-invasive, clinical alternative to ABPI for assessing the presence of significant arterial diseases.

Figure 2.5 Kaplan-Meier survival (%) for 1446 CHS patients with prevalent CVD, by categories of ABPI level (ABPI <0.8, n=258; ABPI 0.8 to <0.9, n=101; ABPI 0.9 to <1.0, n=193; ABPI 1.0 to <1.5, n=894) (Newman et al., 1999).

To overcome the limitations of ABPI, several authors have suggested the use of TBPI to assess PAD (Bonham, 2006; Hoyer, Sandermann, & Petersen, 2013; Mayfield et al., 1998; Young, Adams, Anderson, Boulton, & Cavanagh, 1993), because digital arteries are less affected by calcification. This study compared the use of ABP and TBPI in measuring peripheral blood pressure. Similarly to ABPI, TBPI is a non-invasive method that can be used to assess lower
extremity arterial circulation by comparing systolic pressure in the toe to the systolic brachial pressure in the arm. Toe pressure measured with plethysmography has been considered to be a more accurate reflection of foot blood supply (Duprez, Missault, Van Wassenhove, & Clement, 1986). TBPI is more effective than ABPI for the identification of peripheral occlusive arterial disease in a diabetic population with peripheral neuropathy (Williams, Harding, & Price, 2005). Brooks et al. (2001) showed a progressive rise in TBPI along with ABPI up to the value of 1.3. Above the ABPI value of 1.3, TBPI was reported to decrease. TBPI has been shown to correlate with novel cardiovascular risk factors such as serum inflammatory markers in DM (Aso et al., 2004). However, no study has found a correlation between oxidative stress markers and TBPI as yet. The present study has associated both ABPI and TBPI with novel cardiovascular risk markers such as chronic inflammation and oxidative stress.

**Section summary:** Several studies have used ABPI as a tool for diagnosing vascular diseases in diabetic populations, but TBPI has been advocated as being more effective than ABPI in measuring peripheral vascular function. Oxidative stress and chronic inflammation are the two main dimensions affecting vascular and endothelial functions; however, no study has investigated the association between oxidative stress and TBPI. Also, there is very limited information regarding inflammation and TBPI. Furthermore, it would be valuable to determine how these peripheral vascular disease markers are affected by altered haemorheology given that haemorheology, itself, is implicated in altered microcirculation.
2.6 Summary of literature review

MetS is the cluster of multiple, interrelated risk factors of metabolic origin that appear to promote the development of CVD (Jiang et al., 2014). The main factors that seem to promote the development of CVD in MetS include obesity, dyslipidaemia and insulin resistance (Grundy et al., 2005). Multiple biochemical, metabolic, and signal transduction pathways contribute to insulin resistance in MetS. MetS is associated with several impairments in microcirculation and macrovascular dysfunction (Czernichow et al., 2010; Gyawali, Richards, Tinley, & Nwose, 2014). It has been demonstrated that these vascular dysfunctions increase peripheral vascular resistance and blood pressure (Jonk et al., 2007). Several factors, such as chronic inflammation, oxidative stress and endothelial dysfunction, have been put forward for the impairment of microcirculation in MetS (Serné et al., 2007). Haemorheology, the important dimension that determines erythrocyte flow properties, has not been given much importance in the literature regarding its possible effect on microcirculation. Several studies have associated MetS and its components with altered haemorheology. However, the precise mechanistic link between MetS and altered haemorheology remains unclear. Oxidative stress and inflammation could be the possible link (Gyawali, Richards, Hughes, & Tinley, 2014; Gyawali, Richards, Nwose, & Bwititi, 2012). A better understanding of the pathophysiology of the syndrome may lead to new therapeutic approaches. It is therefore of great importance to unravel the underlying mechanisms. This section has reviewed the evidence for an association of MetS with haemorheology taking oxidative stress and inflammation into account. Impaired microcirculation due to altered haemorheology could be a possible link between obesity, insulin resistance, and CVD.
Chapter 3: Materials and Methods

Chapter outline

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3.1 Research participants
3.1.1 Recruitment of the research participants

Volunteer participation was requested from the twin border cities of Albury (NSW) and Wodonga (Victoria) in Australia. Participants were recruited from Albury-Wodonga region through advertisements in a local newspaper, news channel, Charles Sturt University (CSU) What’s New and Community Engagement and Wellness Centre (CEW) notice board.

Information about the study was first published in CSU NEWS online on 16th April, 2013. The local newspaper of the Albury-Wodonga region, Border Mail, also published the same information, requesting the volunteer participation of the local residents.

The local television news channels, WIN NEWS and PRIME NEWS also covered the news of the study and asked for the volunteers. The chief investigator was interviewed by ABC Goulburn Murray on-line radio station on 30th of April, 2013 regarding the study. The principal supervisor Dr. Ross S Richards was also interviewed by the two television news channel; ABC NEWS and WIN NEWS. In an interview, He clarified the objectives of the study and asked for the participation of some more volunteers. Further coverage of the study was carried out by CSU NEWS website under the heading “Does Diabetes change blood cells?”

Participants were sourced from within CSU through advertisement in CSU ‘What’s New’. Further information sheet (attached as appendix) regarding the project was affixed to the notice board of CEW, CSU, Thурgoona campus. The participants responded through phone or email to the advertisements and were requested to visit the Skills ward, Thургоona campus, CSU (Room 204, building 666). The study was further well explained, and if they consented, they were enrolled.
3.1.2 Criteria for the recruitment of research participants

Inclusion criteria

People with diagnosed type 2 DM and/or hypertension (who may or may not be taking medications)

Apparently healthy people as the controls

Exclusion criteria

Pregnant women

Non-ambulatory people

Children with age less than 18 years

3.1.3 Ethical Clearance

The proposal of the study was approved by CSU Human Research Ethics Committee (HREC) (2012/131). The approval letter has been attached to the appendix.

3.1.4 Sample size calculation

By considering mean and standard deviation of relevant studies, required sample size was calculated. Sample size to obtain statistical power of 80% at 95% CI based on a similar study (Sola et al., 2007) was calculated as shown below.

Mean and SD of Elongation index (MetS group) = 56.6±2.43

Mean and SD of Elongation index without MetS group = 57.9±2.20
Effect size calculated from Mean and SD was 0.56. (Effect size was calculated for t-test as Difference in mean / mean of SD in two populations)

So, minimum sample size required per group as obtained from A-priori sample size calculator was 52 (Total 104).

The previous studies that have shown haemorheological measurements in MetS have recruited different number of MetS participants (n=18 in the study of Aloulou et al. (2006), n=19 in the study of Presti et al. (2002). Similarly, with reference to the study of Nwose et al. (2010), which have reported the value of WBV in patients with type 2 DM, the minimum sample size required was 51. The 100 participants was our intended sample size which was likely to produce significant results.

3.1.5 Total Number of participants

One hundred consecutive participants who responded through a phone or email were recruited in the study. The recruited participants were classified into either two groups on the basis of the presence or absence of metabolic syndrome (MetS) or into three groups on the basis of number of risk factors present.

Definition of MetS

The modified National Cholesterol Education programme, Adult treatment Panel III (NCEP ATP III) definition (Grundy, Brewer Jr, et al., 2004) was used to define the state of MetS in this study.
Participants having three or more risk factors among five listed below were classified to have MetS.

1. Abdominal obesity (waist circumference >102 cm for men, >88 cm for women)

2. Serum triglycerides ≥1.7 mmol/L or drug treatment for elevated triglycerides

3. Serum HDL-cholesterol <1.04 for men and <1.29 for women or drug treatment to increase HDL-cholesterol

4. Fasting blood glucose level ≥ 5.6 mmol/L or drug treatment for elevated glucose or diagnosed diabetes

5. Systolic blood pressure ≥130 mmHg, diastolic blood pressure ≥85 mmHg or on hypertensive treatment.

3.1.6 Classification of the participants (n=100) on the basis of the above MetS definition
(Figure 3.1)

Without MetS

Group I: n=31. The participants in this group did not have any of the five risk factors used to define MetS and are apparently healthy.

Group II: n=33. The participants in this group meet either one or two of the above mentioned five risk factors.
‘Group I’ and ‘Group II’ participants did not fulfil three of the five above mentioned criteria. So, ‘Group I’ and ‘Group II’ participants did not have MetS.

With MetS

**Group III**: n=36. The participants in this group are classified as having MetS as each one fulfils either three, four or all five of the above mentioned criteria (risk factors) to define MetS.

![Figure 3.1](image_url)

**Figure 3.1** A graph showing distribution of participants in three different groups as per the number of MetS components present (group I: not any risk factors present, group II: one or two risk factors present, group III: three or more risk factors present, MetS)

### 3.1.7 Classification of the participants on the basis of the lipids parameters

The recruited participants were also classified into two groups on the basis of absence or presence of dyslipidaemia (normolipidaemic Vs dyslipidaemic.) Among the 100 recruited participants, one did not have complete lipid profile data. Hence, the remaining 99 participants were re-grouped into two groups. Normolipidaemic group constituted 29 participants whereas dyslipidaemic group constituted 70 research participants. For classification, NCEP-ATP III guideline was referred. According to NCEP ATP-III guideline, hypercholesterolaemia was
defined as total cholesterol > 5.1 mmol/L, increased LDL-C as > 2.5 mmol/L, hypertriglyceridaemia as > 1.7 mmol/L and decreased HDL-C as < 1.03 mmol/L (Expert Panel on Detection Evaluation Treatment of High Blood Cholesterol in Adults, 2001). Dyslipidaemia was defined by presence of one or more than one abnormal serum lipid concentration.

3.2 Specimens

3.2.1 Venous blood sample: Twenty (20 mL) of venous blood was collected from median antecubital fossa from all the research participants adhering to standard operating procedures for blood collection. Blood was collected in six different vials.

3.2.2 Urine sample: Mid-stream urine samples were collected from all the research participants using the nearby toilet facilities for the estimation of urinary isoprostanes and creatinine.
Table 3.1: Blood collection tubes and anticoagulants

<table>
<thead>
<tr>
<th>Tubes</th>
<th>Amount collected</th>
<th>Requirement</th>
<th>Tests applicable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vial containing 2 mL of glutaraldehyde</td>
<td>2-4 drops (directly from the needle)</td>
<td>Whole blood</td>
<td>Scanning Electron microscopy</td>
</tr>
<tr>
<td>Plain tube with clot activator</td>
<td>7 mL</td>
<td>Serum</td>
<td>Lipid profile tests, hs-CRP</td>
</tr>
<tr>
<td>Sodium fluoride tube</td>
<td>1 mL</td>
<td>Plasma</td>
<td>Fasting blood glucose (FBG)</td>
</tr>
<tr>
<td>Sodium citrate tube</td>
<td>2 mL</td>
<td>Plasma</td>
<td>D-dimer</td>
</tr>
<tr>
<td>Heparinised tube</td>
<td>5 mL</td>
<td>Washed cells, plasma, haemolysate</td>
<td>Erythrocyte reduced Glutathione, superoxide dismutase</td>
</tr>
<tr>
<td>EDTA tube</td>
<td>4 mL</td>
<td>Whole blood</td>
<td>Haemorheological tests</td>
</tr>
</tbody>
</table>

Standard operating procedures for the urine collection

1) A sterile and labelled (coded) urine specimen container was given to the research participants.

2) The participants were requested firstly to cleanse the genitals using a sterile saline wipe.
3) The participants were requested to collect urine after the first few moments of urination (midstream urine)

4) After collection, the containers were placed in the ice before taking to the laboratory

5) Urine samples were taken to the research laboratory for an appropriate storage (-80°C) as soon as possible after collection.

Figure 3.2 Flow diagram highlighting the steps performed for participants recruitment and data collection
3.3 Anthropometric and blood pressure measurements

3.3.1 Standard Operating procedures for the measurement of toe brachial pressure index (TBPI) using a SysToe (ATYS medical)

Principle of the method (Atys Medical)

(refer to: http://www.medexa.se/wp content/uploads/2010/02/Atys-SysToe-broschyr.pdf)

SysToe uses photoplethysmography (PPG) analysed by a unique patented algorithm designed by Atys Medical. Light from an emitting diode (LED) is directed towards the skin where it is absorbed and scattered in tissues. An adjacent photo-sensor detects the backscattered light whose variation is related to changes of blood volume in the underlying tissues. The PPG sensor is placed on the distal pad of the toe and the occlusion cuff is wrapped on the proximal part of the toe. The occlusion cuff is inflated automatically to a pressure sufficient to stop blood flow, then deflated slowly at a controlled rate. During deflation, resumption of blood flow downstream from the cuff is detected by the PPG sensor. The cuff pressure at this time is the toe systolic pressure.

Procedures

1) The participants were requested to lie down flat on the bed with their heart and feet at the same level.

2) The blood sample was obtained from the participants before measuring blood pressure.

3) Brachial systolic pressure was measured with the automated BP machine (Welch Allyn®) in the clinic, from both arms.
4) Participants were advised that they were not allowed to talk during the subsequent Systoe testing.

5) The participants were given at least ten minutes time to rest in a supine position before measurement procedures.

6) An occlusion cuff was wrapped at the base of the great toe (the size of the occlusion cuff selected depended on the sex or size of the great toe)

7) Double sided ring tape (supplied) was applied on the sensor of sensor cuff as directed by the manual.

8) The sensor cuff was placed on the pulp extremity of the toe as suggested by manual

9) Systoe was switched on and all the further steps were done exactly as directed by the manual and instrument

10) Brachial systolic pressure was entered on the machine pressing on Bra.P.

11) The displayed signal was pulsed or flat. The measurement was automatically performed upon pressing START.

12) STOP was pressed when there was a definite and confirmed increase of the sensor signal.

13) The opposite screen was displayed. If the position of the vertical cursor was at the foot of the upslope of the sensor signal, VALID was pressed.

14) The toe systolic pressure and TBPI were noted as displayed on the machine.

15) The whole procedures were repeated on the great toe of a next leg.

16) The higher of the two values was taken into account for a study purpose.
3.3.2 Standard operating procedures for the measurement of ankle brachial pressure index (ABPI)

**Principle of the method**

The ankle brachial pressure index (ABPI) calculation is based on the doppler principle, which states that the frequency of a sound emitted or reflected from a moving object varies with the velocity of the object. The ratio of arm and ankle systolic pressure (the highest reading) eliminates systolic pressure variation (Greenland et al., 2000).

**Equipment and supplies**

Doppler Ultrasound with 8-megahertz peripheral probe (Hadeco, Inc.)

Portable manual blood pressure cuff

Conducting ultrasound gel

Automated BP machine (Welch Allyn)

**Procedures**

1. The participants were given ten minutes time to rest in a supine position before measurement

2. Brachial systolic pressure was measured with the automatic BP machines (Welch Allyn) in the clinic, from the both arms

3. BP cuff was applied 1 cm above the lateral malleolus

4. Dorsalis pedis pulse in the feet was located

5. Dorsalis pedis pulse was palpated, and the gel was applied over the pulse
6. Doppler was turned on and the doppler probe was held at 45 to 60-degree angle towards the blood flow

7. Doppler probe was moved slowly through the gel in a circular motion until a pulse sound was heard.

8. BP cuff was inflated (at the rate of 2-3 mmHg) until the pulse sound disappeared and then inflated 10 – 20 mmHg more; Cuff was not inflated past 200 mmHg as it may dislodge plaques in blood vessels.

9. Cuff was gradually deflated until the arterial sound returned. When the sound was heard, cuff was completely deflated. The pressure at which the sound returned was recorded.

10. The whole procedures were repeated on the other ankle. Higher systolic ankle reading was used to calculate the ABPI.

Calculation

The higher of the two ankle pressures

\[
\text{ABPI} = \frac{\text{The higher of the two ankle pressures}}{\text{The higher brachial pressure of the two arms}}
\]

3.3.3 Standard operating procedures for the measurement of participant’s height

1) The participants were asked to remove their footwear and headgear if any.

2) They were requested to stand straight with their feet together facing towards chief investigator. (Measurement apparatus was positioned on a firm surface against the wall)

3) The measurement apparatus was moved gently down onto the head of participants
4) Height in centimeters at the exact point was recorded and converted to metres.

3.3.4 Standard operating procedures for the measurement of participant’s weight

1) A measurement scale was put on a firm, flat surface

2) The participants were requested to remove their footwear and anything (such as key, purse, etc.) from their pocket.

3) They were requested to step onto scale and stand still facing forward

4) Weight was recorded by the chief investigator in kilograms.

3.3.5 Standard operating procedures for the measurement of participant’s waist circumference

1) The participants were requested to stand with their feet together

2) Waist circumference was measured from the upper margin of the posterior iliac crest (hip bone) at the end of a normal expiration directly above the skin

3) A measuring tape was kept snug, but not tight enough to cause compression of the skin.

4) A measurement was recorded at the nearest 0.1 cm.
3.4 Quantitative analysis in the research laboratory

3.4.1 Sample preparation, separation and storage

Blood was placed on ice before transportation to the pathology and research laboratory. In the research laboratory, heparinised blood samples were centrifuged at 3000 RPM for five (5) minutes using bucket centrifuge (Universal® 32 R Hettich Zentrifugen, Germany) to obtain plasma. The plasma sample so obtained along with the urine sample were separated in 1.5 mL Eppendorf tubes, labelled and stored at -80°C in an ultra low-temperature freezer until tests were performed.

Haemolysate was prepared from packed red blood cells. One mL of packed red blood cells were mixed with four mL of ice cold water and centrifuged at 3000 RPM for nine minutes to obtain haemolysate. The haemolysate was also stored in 1.5 mL Eppendorf tubes at -80°C in an ultra low temperature freezer until tests were performed. Remaining of the packed cells was washed three times in Dulbecco’s phosphate buffered saline to obtain washed red blood cells.

Plain tube, sodium fluoride tube and sodium citrate tube were taken to Dorevitch pathology laboratory for the analysis of lipid profile, hsCRP, d-dimer and blood glucose level.
3.4.2 Standard operating procedure for the estimation of whole blood viscosity (WBV)

Principle of the method

WBV measurement was carried out using a Brookfield DV-II+ programmable viscometer. This viscometer measures blood viscosity at different shear rates. The principle of the operation of the viscometer is to drive a spindle (which is immersed in the test fluid) through a calibrated spring. The viscous drag of the blood against the spindle (CP40) is measured by the spring deflection. Spring deflection is measured with a rotary transducer. The measurement range is determined by the rotational speed of the spindle (CP40), the container the spindle is rotated, and the full scale torque of the calibrated spring.

Specimen

40% haematocrit (±1%) adjusted EDTA blood

Haematocrit of the blood samples was adjusted to 40±1% by adding or removing autologous plasma of the participants. Haematocrit of the samples was assayed by microhaematocrit centrifugation (Capspin Sorvall®).

Procedures

The viscosity of all the blood samples was measured at 37°C within one and half hours of blood collection.

The method as recommended by the manufacturers and detailed in the instrument manual was followed. In brief,

1) The viscometer was switched on
2) Using the bubble on the top of the viscometer, it was confirmed that the viscometer was levelled.

3) Using the keypad (up arrow) on the viscometer, external mode was selected and all the command was given externally through a connected laptop computer.

4) Prior to the measurement, ‘Gap’ was set by following the manual instructions (Setting the Gap)

5) When the gap was set, sample cup was removed from its original position and the viscometer was auto zeroed through software in the laptop computer.

6) 500 µL of whole blood was pipetted into the sample container and the sample container was placed back in its original position (attached to the viscometer through shaft).

7) Through a specific pre-created protocol (speed and shear rate was preselected through this protocol), command was given to measure the viscosity at a different shear rates (from 0.1 to 200 s⁻¹).

8) WBV at the different shear rates was noted.

9) The sample cup was removed at the conclusion of the test.

10) The cup and spindle were cleaned with the help of alcohol swab and distilled water after each test.

Note: Though viscosity was measured at the shear rates of 0.1, 11, 22, 45, 55, 66, 77, 89, 100, 150 and 200 s⁻¹, viscosity readings above shear rate of 45 and above was considered for research purpose. At these shear rates; percentage torque was between 10 to 100%. All the WBV values are given at the shear rate of 150 s⁻¹ unless specified.
Calibration

Viscometer was calibrated using the silicone viscosity standard (viscosity of 9.7 mPa.s at 25°C). A calibration template provided by the company was used to check the validity of the instrument.

3.4.3 Standard operating procedure for the measurement of erythrocyte deformability

Principle of the method (Caimi & Lo Presti, 2004; Martínez, Vayá, Server, Santaolaria, & Aznar, 1998; Simmonds et al., 2010)

Erythrocyte deformability measurements were carried out using a RheoScan-And 300 system (RheoMeditech; Inc., Korea). The operating principle of the instrument for the measurement of erythrocyte deformability was based on Laser diffraction. The light source was a red diode laser (wavelength: 635 nm). Erythrocytes in a medium were deformed by a range of shear stress (0.5-20 Pa). When a laser beam passed through the erythrocytes suspended in a viscous medium (poly vinyl pyrrolidone), a diffraction pattern of light was obtained. This diffracted light was projected onto the screen. The image analysis programme determined the elongation index (EI) to represent the cell deformability of the sample. EI was defined according to the formula \((L - W)/(L + W) \times 100\), where \(L\) = length of the major axis in the ellipsoidal diffraction image and \(W\) = length of the minor axis in the ellipsoidal diffraction image.
Requirements

1) Disposable test Kit (supplied)

The RheoScan-And test kit is a disposable test kit that consists of a sample chamber, microchannel, waste sample chamber and a rubber cap. The kit is made of transparent plastic. The dimension of the microchannel was 0.2 mm height and 40 mm length.

2) Poly Vinyl Pyrrolidone (PVP) Solution (0.6 mL each, supplied)

This is a viscous solution (MW- 360,000) dissolved in phosphate buffered saline solution (final viscosity: 30-32 mPa.s at 37°C, Osmolality: 300 mOsm/kg).

Specimen

40% haematocrit (±1%) adjusted EDTA blood pre-warmed to 37°C in water bath

Haematocrit of the blood samples was adjusted to 40±1% by adding or removing autologous plasma. Haematocrit of the samples were assayed by microhaematocrit centrifugation (Capspin Sorvall®).

Procedures

Measurements were performed in duplicate for each sample, within one and half hours of blood collection. The method as recommended by the manufacturers and detailed in the instrument manual was strictly followed. In brief,
1) Six (6) µL of EDTA blood was aspirated from the tube and mixed with 0.6 mL of PVP solution (provided in a separate vial). Blood was gently mixed with the PVP solution.

2) 0.5 mL of mixed blood (blood + PVP solution) was transferred in the provided test kit. (The formation of bubbles was avoided as much as possible)

3) Test kit was loaded into RheoScanD-300 with rubber cap side on the right.

4) Command was given to the instrument through connected laptop for the measurement of erythrocyte deformability.

Measurement indices (Baskurt et al., 2009; Simmonds et al., 2012)

All the deformability measurements were performed in the duplicate to identify following parameters that describe erythrocyte deformability.

- $E_{\text{Imax}}$ (Elongation Index maximum): This is the maximum erythrocyte elongation index at the infinite shear stress
- SS$_{1/2}$: This is the shear stress required for the half of maximal deformation
- EI@3 Pa: This is the elongation index at shear stress of 3 Pa.

3.4.4 Standard operating procedure for the measurement of erythrocyte aggregation

Principle of the method (Hou & Shin, 2008)

Erythrocyte aggregation measurements were carried out using a RheoScan-And 300 system (RheoMeditech; Inc., Korea). The operating principle of the instrument for the measurement of erythrocyte aggregation is based on light transmission. The transmitted light was detected by photodiode and recorded over 120 seconds and analysed for aggregation indices.
A sample chamber, which was open to the atmosphere, was filled with a test fluid (0.5 ml). When the valve between the vacuum generating system and the waste chamber was opened, the test fluid started to flow through the microchannel and to be collected in the waste chamber as it was being driven by the pressure differential. The test fluid stopped flowing once the pressure differential reached equilibrium with a pressure head. While the test fluid was flowing, pressure differential and backscattered light intensity were simultaneously measured and analysed. While the blood was flowing through the microchannel, a laser beam emitted from the laser diode traversed the diluted erythrocyte suspension and was scattered by the erythrocytes in the volume. The backscattered light was captured by two photodiodes that were linked to a computer. When high pressure differential was initially applied, strong shear flow occurred through the microchannel and the erythrocyte aggregates started to disaggregate as was indicated by the corresponding increase in light intensity. As the pressure differential decreased exponentially, the shear flow also decreased and the disaggregated erythrocytes tended to re-aggregate. Thus, the corresponding light intensity tended to decrease. The initial increase of backscattered light was caused by the disaggregation of erythrocytes due to high-shear flow, and the decrease of backscattered light was a result of the re-aggregation of erythrocytes that’s associated with low-shear flow (Zhao, Wang, & Stoltz, 1999).
Requirements

1) Disposable test Kit (supplied)

The RheoScan-And test kit is a disposable test kit, that consists of a sample chamber, microchannel, waste sample chamber and a rubber cap. The kit is made of transparent plastic. The dimension of the microchannel is 0.2 mm width and 40 mm length.

Specimen

40% haematocrit (±1%) adjusted EDTA blood pre-warmed to 37°C in water bath

Haematocrit of the blood samples was adjusted to 40±1% by adding or removing autologous plasma. Haematocrit of the samples were assayed by microhaematocrit centrifugation (Capspin Sorvall®).

 Procedures

Measurements were performed in duplicate for each sample, within one and half hours of blood collection. The method as recommended by the manufacturers and detailed in the instrument manual was strictly followed. In brief,

1) Test kit was filled with 0.5 mL of blood, without creating any bubbles.

2) Test kit was loaded into RheoScanD-300 with rubber cap side on the right.

3) Command was given to the instrument through connected laptop computer for the measurement of erythrocyte aggregation.
Measurement indices

All the aggregation measurements were performed in duplicate to identify following two parameters that describe the erythrocyte aggregation.

Critical time and critical stress: Critical time is the maximum point of backscattered light (figure 3.3). This point indicates the starting point of the RBC re-aggregation, and this is strongly dependent on both shear stress and time. The time and shear stress values corresponding to the maximum point of backscattered light are defined as the critical time and the critical shear stress. Before and after these values of critical time and critical shear stress, there is a critical transition period from dis-aggregation to re-aggregation of the erythrocytes that’s associated with a decreasing shear flow

Note: The lesser the critical time, the faster is the erythrocyte aggregation process.

The higher the critical stress, the faster is the erythrocyte aggregation process.
Figure 3.3 (Hou & Shin, 2008) The graph showing critical time and critical stress point.
3.4.5 Standard operating procedures for scanning electron microscopy (SEM)

Principle of the method

Scanning electron microscope (JCM 5000, Benchtop SEM, Neoscope) was used to study the morphology of the erythrocytes. The main components of this microscope were an electron gun, lenses, and a specimen chamber which was evacuated to a high vacuum with the evacuation system. Electron beam generated with an electron gun was focused to the specimen. Specimen surface was scanned by the electron beam in X-Y directions. By the scanning of an incident electron beam on the specimen, secondary electrons and backscattered electrons were emitted from the specimen surface. These electrons were detected as a signal, and the morphology of the specimen surface was converted in three dimensional images on the display.

Before microscopy, the specimen was coated with a thin layer of gold. This was done using a K550X sputter coater. The layer deposited was typically 10 to 20 nanometers thick, and nearly evenly coated the surface of the specimen, faithfully reflecting the surface morphology.

Reagents/ materials

1. Cacodylate buffer (0.1 M, pH 7.4): 21.4 g sodium cacodylate (Sigma life Science) was dissolved in 1 liter of deionised water and the pH adjusted to 7.4 with 1M HCl (BDH Analar®)

2. Fixative: 2.5% glutaraldehyde (VWR BDH Prolabo®) in 0.1 M cacodylate buffer
3. Ethanol, absolute, AR grade (VWR BDH Prolabo®)

4. Ethanol (95%): 950 mL absolute ethanol was diluted with 50 mL deionised water

5. Ethanol (70%): 700 mL absolute ethanol was diluted with 300 mL deionised water.

6. Acetone, AR grade (Mallinckrodt Chemicals)

7. No 1 coverslip (ProSciTech) (round): 12 mm diameter

8. Carbon Tabs (ProSciTech)

9. SEM mount Pin (ProSciTech)

10. Carbon/Graphite (ProSciTech)

11. K550X Sputter Coater (Quorum Technologies Ltd, UK)

12. Scanning electron microscope (JCM-5000)
Procedures (Richards et al., 2000)

1) 2 to 3 drops of blood immediately after collection was fixed in glutaraldehyde fixative at the phlebotomy site. This was mixed gently by inversion. Any further processing of the mixture was done as per the convenience of the chief investigator.

2) The fixed erythrocytes were re-suspended in the fixative and one ml of the suspension was then transferred to a centrifuge tube, which was centrifuged (Universal® 32 R Hettich Zentrifugen, Germany) at 800 RPM for four minutes.

3) The fixative was removed and replaced with cacodylate buffer. The cells were washed (800 RPM for four minutes) in two changes of buffer.

4) The erythrocytes were then dehydrated by washing ((800 RPM for four minutes) twice in 70% ethanol, twice in 95% ethanol, twice in absolute ethanol and twice in acetone and then resuspended in acetone.

5) The final amount of acetone used for re-suspension depends on the size of erythrocyte pellet in the test tube

6) Twenty (20) µl of suspension was placed on two coverslips and spread by gentle coverslip movement (coverslip placed between thumb and a finger and rotated gently 2/3 times and then allowed to dry.)

7) Monolayer distribution of cells was confirmed by 10X light microscopy (Olympus).

8) Coverslips were mounted onto carbon tabs (eccentrically) which were already placed onto SEM mount pin type

9) Coverslips were stored in a desiccator containing self-indicating silica gel (blue when dry, pink when moist)
10) Since coverslips were placed eccentrically onto a carbon tab, small portion of coverslip was outside the carbon tab/SEM mount pin.

11) The lower part of the portion of the coverslip, which was projected outside the mount pin, was coated with the Carbon/Graphite.

12) The specimens were inserted into the sample holder which in turn was placed in the sputter coater.

13) Sputter coater was started, and the specimen was coated with a thin layer of gold.

14) Specimens were transferred to the SEM for analysis.

15) After the image was obtained, appropriate areas of the specimen were photographed using the instrument software.

16) The erythrocytes in each photograph were examined, classified and counted.
3.4.6 Standard operating procedures for the measurement of Glutathione (Beutler, Duron, & Kelly, 1963)

Principle of the method

Oxidized glutathione (GSSG) was reduced enzymically to reduced glutathione (GSH). When 5,5'-dithiobis-(2-nitro benzoic acid) (DTNB) was added to this reduced sulphhydryl group, yellow complex anion was formed, the absorbance of which was proportional to the concentration of the compound. The peak of absorbance was at 412 nm. Mixed disulphide was formed along with coloured complex. This disulphide reacted with further quantities of GSH to liberate another ion and GSSG, which then re-entered the cycle (Owens & Belcher, 1965).

![Figure 3.4](image.png) Figure showing conversion of DTNB to yellow coloured anion.

Reagents

1) Precipitating solution

8.35 g glacial metaphosphoric acid (Acros Organics)

1.0 g ethylene diamond tetra-acetic acid, dipotassium salt (Chem Supply)

150.0 g sodium chloride

Deionised water to 500 mL
2) Phosphate solution

42.6 g disodium hydrogen orthophosphate (0.3M) (VWR BDH Prolabo®)
Deionised water to 1L
Stored at room temperature

3) DTNB reagent

40 mg 5,5'-dithiobis-(2-nitro benzoic acid) (Chem Supply)
10 g/L sodium citrate to 100 mL
Stored at 4 ºC

4) Glutathione standard

50 mg reduced glutathione (Acros Organics)
Deionised water to 100 mL
Prepared fresh daily

Specimen Preparation

3.0-4.0 mL heparinised whole blood was washed three times in Dulbecco's phosphate buffered saline (Sigma Life Science) and re-suspended in Dulbecco's phosphate buffered saline.
Haematocrit of the final suspension was assayed by microhaematocrit centrifugation (Capspin Sorvall®).

**Procedures**

Sequential addition of the reagents and the measurement of absorbance is shown in the chart below.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionised water</td>
<td>2 mL</td>
<td>1.8 mL</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>0.2 mL</td>
<td>-</td>
</tr>
<tr>
<td>Precipitating solution</td>
<td>3 mL</td>
<td>3 mL</td>
<td>3 mL</td>
</tr>
<tr>
<td>Washed red cells</td>
<td>-</td>
<td>-</td>
<td>0.2 mL</td>
</tr>
</tbody>
</table>

Stand 5 minutes. Mixed by inversion in between. Filter through Whatman number 285 filter paper.

| Filtrate                  | 2.0 mL| 2.0 mL | 2.0 mL |
| Phosphate solution        | 8.0 mL| 8.0 mL | 8.0 mL |

Mix by inversion then read absorbance at 412 nm against the blank (OD1).

| DTNB                      | 1.0 mL| 1.0 mL | 1.0 mL |

Mix by inversion and then read the absorbance at 412 nm against the blank (OD2).
### Calculation

\[
\text{OD2} - \text{OD1 (test)} \times \text{Value of standard (50mg/100mL)}
\]

\[
\text{OD2} - \text{OD1 (std)} \quad \text{Hct (washed cells)}
\]

### 3.4.7 Standard operating procedure for the estimation of superoxide dismutase (SOD)
(Cayman Chemical)

**Principle of the method**

Xanthine oxidase converted xanthine into uric acid and superoxide anion in the presence of oxygen. Generated superoxide converted colourless tetrazolium salt into the coloured formazan dye. This later reaction was inhibited by SOD enzyme. 50% inhibition activity of SOD was determined from the cayman kit colorimetrically. One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

**Figure 3.5** Figure showing the conversion of colourless tetrazolium salt to the coloured formazan dye using the generated superoxide free radical.
Reagents/ materials

- Kit supplied by the Cayman company (item no. 706002)
- Plate reader (Multiskan FC Microplate Photometer)

Specimen

Haemolysate (final dilution 1:100)

Steps for the preparation of haemolysate

- Erythrocytes (collected in the heparinised tube) after removing plasma and buffy coat were lysed in four times its volume of ice cold distilled water.
- This was then centrifuged at 3000 RPM for 15 minutes at 4°C
- Supernatant was collected and stored at -80°C until estimation.

Procedures

Instructions provided by the company were strictly followed. In brief, pipetting procedures are as follows. All the samples and standards were run in duplicate.
<table>
<thead>
<tr>
<th>Reagents</th>
<th>Sample</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted radical detector</td>
<td>200 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>10 µL</td>
</tr>
<tr>
<td>Diluted sample</td>
<td>10 µL</td>
<td>-</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>20 µL</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

Incubate 20 minutes at the room temperature on a shaker

Read the absorbance at 450 nm using the plate reader

Calculations

1) Average absorbance of each standard and sample was calculated.

2) Absorbance of Std A was divided by itself and Std A absorbance’s was divided by the absorbance of all the other standards and samples to yield the linearised rate (LR)

3) To obtain a standard curve, LRs of all standards were plotted against the final SOD activity of standard.

4) SOD activities of the samples were obtained using the equation obtained from the linear regression of the standard curve

\[
SOD (U/mL) = \left(\frac{\text{sample LR} - \text{y-intercept/slope}}{0.23 mL/0.01 mL}\right) \times \text{sample dilution}
\]

\[
\text{sample LR} = \left(\frac{\text{sample absorbance}}{\text{standard absorbance}}\right)
\]
3.4.8 Standard operating procedure for the estimation of 15-isoprostanes F_{2t} (Northwest Life Science Specialities, LLC)

Principle of the method
This assay utilised a competitive enzyme-linked immunoassay (ELISA) strategy. Prior to assaying, all samples were treated with an enhancing reagent. After treatment with enhancing reagent, 15-isoprostane F_{2t} in the samples or standards was allowed to compete with 15-isoprostane F_{2t} conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for 15-isoprostane F_{2t} coated on a microplate. Later TMB substrate addition resulted in a blue colour development that was inversely proportional to the quantity of 15-isoprostane F_{2t} in the original samples or standards. The addition of an acid stop solution caused a colour change to yellow where absorbance was read at 450 nM.

Reagents/ Materials
- Kit supplied by NWLSS™ (Product NWK-ISO002)
- Plate reader (Multiskan FC Microplate Photometer)
- 3 molar sulphuric acid

Specimen
- Urine (final dilution 1:8)
- Urine samples were divided into aliquots and kept frozen at -80°C until analysis
Procedures

Instructions provided by the company were strictly followed. In brief, pipetting procedures were as follows. All the samples and standards were run in duplicate.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Sample</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted urine</td>
<td>100 µL</td>
<td>-</td>
</tr>
<tr>
<td>Standards (S0-S7)</td>
<td>-</td>
<td>100 µL</td>
</tr>
<tr>
<td>Diluted HRP conjugate</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

Incubate plate for 2 hours at room temperature

Wash plate with 300 µL of wash buffer three times

TMB substrate              | 200 µL       | 200 µL     |

Incubate plate for 20-40 minutes at room temperature

3M Sulphuric acid          | 50 µL        | 50 µL      |

Read at 450 nm using plate reader.

Calculations

Average absorbance of each standard and sample was calculated

Calculations template provided by the company was used to calculate the value

Urinary 15-isoprostanes $F_{2\alpha}$ was divided by the urinary creatinine and values were expressed as pg/mmol
3.4.9 Standard operating procedure for the estimation of urinary creatinine (Cayman Chemical Company)

Principle of the method

This assay was based on the Jaffé’s reaction, wherein a yellow/orange colour formed when the metabolite was treated with alkaline picrate. The colour derived from the creatinine was then destroyed at acidic pH. The difference in colour intensity measured at 500 nm before and after acidification was proportional to the creatinine concentration. The sample creatinine concentration was determined using a creatinine standard curve.

Reagents/ Materials

- Kit supplied by the cayman chemical company
- Plate reader (Multiskan FC Microplate Photometer)

Specimen

- Urine (final dilution 1:15)
- Urine samples were divided into aliquots and kept frozen at -80°C until analysis

Procedures

Instructions provided by the company were strictly followed. In brief, pipetting procedures were as follows. All the samples and standards were run in duplicate.
Calculations

- Average absorbance of each standard and sample was calculated.
- Average final absorbance was subtracted from the average initial absorbance (corrected absorbance).
- Average corrected absorbance of Std A was subtracted from itself and from all other standards and samples (adjusted absorbance).
- Adjusted absorbance of the standards was plotted as a function of the final concentration of creatinine as provided by the company.
- Creatinine concentrations of the samples were calculated using the equation obtained from the linear regression of the standard curve.

Creatinine = \[ \frac{[(\text{Sample average absorbance} - \text{y-intercept})/ \text{slope}]}{X \text{ dilution factor}} \]
3.5 Statistical analysis

All the quantitative results were entered into and analysed by IBM SPSS Statistics software version 20.

3.5.1 Distribution of data: Normality of the data was tested by Shapiro-Wilk test (Shapiro & Wilk, 1965). The null hypothesis of the test was that the population was normally distributed. If the $P$-value was less than 0.05 (the chosen alpha level, 95% Confidence interval (CI)), then the null hypothesis was rejected (i.e. it was concluded that the data were not from a normally distributed population). If the $P$-value was greater than 0.05 (the chosen alpha level), then it was concluded that the data came from a normally distributed population. The distribution of data (parametric or non-parametric) in different groups analysed (normolipidaemia Vs dyslipidaemia or group I, II or III) has been specifically mentioned in the results section (refer to Table 4.1 and 4.2). Parametric tests, such as, the independent sample t-test, ANOVA and Pearsons correlation, were used to analyse the normally distributed data, whereas in non-normal data, non-parametric tests such as Mann-whitney U test and Kruskal wallis were used for analysis. It is suggested to use non-parametric tests for non-normal data rather than using parametric test after attempting to normalise the data (Leech & Onwuegubuzie, 2002).

3.5.2 Independent sample t-test and Mann whitney U test

Independent sample t-test was used to compare the mean of data (normal distribution) between two different groups. Assumption of homogeneity of variance was tested by Levene’s test for equality of variances. If the assumption of homogeneity of variance is violated ($P$
value of levene’s test < 0.05) than Welch t-test was used to interpret the results. The effect size (d) was also calculated for the t-test using the following formulae

\[
d = \frac{|M_1 - M_2|}{s_{pooled}}
\]

\[
s_{pooled} = \sqrt{\frac{s_1^2(n_1 - 1) + s_2^2(n_2 - 1)}{n_1 + n_2 - 2}}
\]

Where

- \(M_1\) and \(M_2\) are mean of two different groups
- \(S_1\) and \(S_2\) are standard deviations of two different groups
- \(n_1\) and \(n_2\) are sample size of two different groups

Mann-Whitney U test was used to compare the median of the data (non-normal distribution) between the two different groups (normolipidaemic Vs dyslipideamic). Similarity in the distribution of parameters analysed was assessed by visual inspection. Median score of the parameters in two groups along with U and Z value were reported.

3.5.3 ANOVA (Analysis of Variance) test and Kruskal-Wallis test

ANOVA was used to compare the mean of data (normal distribution) between three groups (group I, group II and group III: refer to 3.1.5). Assumption of homogeneity of variance was tested by Levene’s test for equality of variances. If the assumption of homogeneity of variance
is violated ($P$ value of levene’s test < 0.05) than Welch ANOVA was used to interpret the results. Tukey Post-hoc test was used to describe the difference between two different groups if Assumption of homogeneity of variance was not violated. Games-Howell Post-hoc test was used to describe the difference between two different groups if Assumption of homogeneity of variance was violated. Kruskal-Wallis test was used to compare the median of the data (not normal distribution) between three groups. A Kruskal-Wallis pairwise comparison was done as a Post-hoc test. The pairwise comparisons were performed using Dunns (1964) procedure with a Benferroni correction for multiple comparisons. In the Benferroni correction, the alpha level is reduced first by dividing previously accepted alpha level 0.05 by the number of comparisons. If there are three groups, for example, then, multiple comparisons are made between Group I and II, Group I and III and group II and III. Hence, new accepted alpha level would be $0.05/3=0.016$.

3.5.4 Correlational analysis: Pearson’s (parametric test) or Spearman’s (non-parametric test) correlation was used to find out the correlation between different variables in questions.

3.5.5 Regression analysis: Ordinal logistic regression analysis was run through Generalised Linear Model to find out the association of different groups (group I, Group II or group III) with the highest quartile of WBV (150 s$^{-1}$), critical stress and the lowest quartile of $E_{I_{\text{max}}}$ (categorical variables). In other words, odds of having highest quartile or lowest quartile value in group III in comparison to the group I or II was calculated. Binomial logistic regression was used to find predict the probability that an observation falls into one of two categories of a dichotomous dependent variable (Mets or not MetS) based on one or more independent variables (haemorheological parameters, ABPI and TBPI) that can be either continuous or categorical.
Odds ratio was calculated. **Linear regression analysis** was run in order to find out the association of continuous independent variables and continuous or categorical predictor variables, such as association between haemorheological parameters (continuous) with lipid parameters or the components of MetS.

**3.5.6 Receiver Operating Characteristics (ROC) curve:** **ROC Curve** was constructed to compare the diagnostic value of the parameters.
Chapter 4: Results

4.1 Descriptive statistics, distribution of the biomarkers and statistical tool selection

4.2 Oxidative stress and chronic inflammation in metabolic syndrome

4.3 Dyslipidaemia and altered haemorheology

4.4 Metabolic syndrome and altered haemorheology

4.5 Oxidative stress, chronic inflammation and altered haemorheology

4.6 Erythrocyte morphology, oxidative stress, chronic inflammation and metabolic syndrome

4.7 Peripheral vascular disease markers: ABPI and TBPI and its association with haemorheology, oxidative stress and chronic inflammation

4.8 Association of altered haemorheology, peripheral vascular diseases markers and novel cardiovascular risk factors with metabolic syndrome
4.1 Descriptive statistics, distribution of the biomarkers and statistical tool selection

One hundred participants were recruited in the present study to determine the effect of dyslipidaemia and MetS on the rheological properties of blood. The participants were divided into three groups based on the number of MetS components present. Participants in Group I (n=31) have ‘0’ component positive; Group II (n=33) have one or two components positive; and Group III (n=36, MetS group) have three or more components positive (refer to 3.1.6). Distribution of biomarkers (normal or not normal) as analysed by Shapiro-wilk test in the three groups is shown in Table 4.1. Demographic characteristics of the participants and descriptive statistics of the biomarkers are shown in Table 4.2a and 4.2b.

Table 4.1: Distribution of biomarkers in three different groups as assessed by Shapiro-wilk tests

<table>
<thead>
<tr>
<th>Normal distribution (P-value &gt;0.05)</th>
<th>Not normal distribution (P-value &lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBV, critical stress and $E_{I_{max}}$</td>
<td>Critical time, $SS_{1/2}$</td>
</tr>
<tr>
<td></td>
<td>Plasma D-dimer, serum hsCRP,</td>
</tr>
<tr>
<td></td>
<td>Erythrocyte SOD, erythrocyte reduced</td>
</tr>
<tr>
<td></td>
<td>glutathione, urinary isoprostanes,</td>
</tr>
<tr>
<td></td>
<td>ABPI and TBPI</td>
</tr>
</tbody>
</table>
Table 4.2a: Demographic characteristic of the participants and mean values (±SD) of MetS components in three different groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (n=31)</th>
<th>Group II (n=33)</th>
<th>Group III (n=36)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>45.4±8.9</td>
<td>56.52±12.9</td>
<td>63.0±12.1</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>10/21</td>
<td>19/14</td>
<td>24/12</td>
<td>-</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>81.16±8.4</td>
<td>95.79±13.23</td>
<td>110.25±15.46</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>166.0±9.0</td>
<td>167.9±6.2</td>
<td>170.8±8.2</td>
<td>0.050</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>67.63±9.27</td>
<td>76.25±13.32</td>
<td>90.14±16.74</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>24.49±2.29</td>
<td>27.02±4.66</td>
<td>30.89±5.50</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>116.2±9.8</td>
<td>127.5±15.1</td>
<td>142.5±19.1</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>FBG (mmol/L)</td>
<td>6.92±2.64</td>
<td>6.06±1.97</td>
<td>4.93±0.41</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.45±0.27</td>
<td>1.43±0.43</td>
<td>1.09±0.22</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>1.09±0.34</td>
<td>1.37±0.62</td>
<td>2.06±0.85</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Current smokers (n)</td>
<td>1</td>
<td>4</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>Antidiabetic medications</td>
<td>-</td>
<td>11</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>Antihypertensive medications (n)</td>
<td>-</td>
<td>13</td>
<td>29</td>
<td>-</td>
</tr>
<tr>
<td>Antilipideamic medications (n)</td>
<td>-</td>
<td>3</td>
<td>12</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4.2b: Demographic characteristics of the participants and summary of the statistical characteristics of the biomarkers in three different groups

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Statistics</th>
<th>Group I (n=31)</th>
<th>Group II (n=33)</th>
<th>Group III (n=36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum hsCRP (mg/L)</td>
<td>Mean±SD</td>
<td>1.13±1.91</td>
<td>2.65±2.76</td>
<td>3.80±3.74</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>0.44</td>
<td>1.25</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0.10 to 9.76</td>
<td>0.10 to 8.84</td>
<td>0.10 to 14.20</td>
</tr>
<tr>
<td>Plasma D-dimer (µg/mL)</td>
<td>Mean±SD</td>
<td>0.38±0.16</td>
<td>0.62±0.93</td>
<td>0.62±0.40</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>0.36</td>
<td>0.39</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0.22 to 0.91</td>
<td>0.23 to 5.39</td>
<td>0.22 to 2.03</td>
</tr>
<tr>
<td>Erythrocyte SOD (U/mL)</td>
<td>Mean±SD</td>
<td>624.7±107.33</td>
<td>579.46±79.48</td>
<td>561.18±92.69</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>630.0</td>
<td>587.69</td>
<td>524.79</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>467.4 to 799.84</td>
<td>467.4 to 733.19</td>
<td>440.69 to 734.46</td>
</tr>
<tr>
<td>Erythrocyte GSH (mg/dL)</td>
<td>Mean±SD</td>
<td>64.60±8.12</td>
<td>60.97±8.92</td>
<td>53.80±6.03</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>65.10</td>
<td>61.80</td>
<td>52.0</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>42 to 76</td>
<td>36 to 77</td>
<td>44 to 65</td>
</tr>
<tr>
<td>Urinary isoprostanes</td>
<td>Mean±SD</td>
<td>71.05±52.11</td>
<td>137.27±88.12</td>
<td>175.63±98.90</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>65.18</td>
<td>144.09</td>
<td>165.71</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>1.01 to 204.66</td>
<td>11.59 to 332.07</td>
<td>41.01 to 388.30</td>
</tr>
<tr>
<td>WBV (mPa.s)</td>
<td>Mean±SD</td>
<td>3.99±0.33</td>
<td>4.10±0.50</td>
<td>4.56±0.36</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>3.94</td>
<td>4.22</td>
<td>4.33</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>3.13 to 4.87</td>
<td>3.20 to 5.80</td>
<td>3.80 to 5.53</td>
</tr>
</tbody>
</table>
Table 4.2b: contd..

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Statistics</th>
<th>Group I (n=31)</th>
<th>Group II (n=33)</th>
<th>Group III (n=36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EImax</td>
<td>Mean±SD</td>
<td>0.561±0.029</td>
<td>0.557±0.022</td>
<td>0.534±0.021</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>0.568</td>
<td>0.562</td>
<td>0.534</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0.506 to 0.614</td>
<td>0.50 to 0.597</td>
<td>0.50 to 0.593</td>
</tr>
<tr>
<td>SS1/2</td>
<td>Mean±SD</td>
<td>2.70±0.49</td>
<td>2.69±0.44</td>
<td>2.85±0.44</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>2.60</td>
<td>2.55</td>
<td>2.90</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>2.0 to 4.0</td>
<td>2.0 to 4.0</td>
<td>2.0 to 4.0</td>
</tr>
<tr>
<td>Critical stress (Pa)</td>
<td>Mean±SD</td>
<td>274.23±48.44</td>
<td>343.48±101.34</td>
<td>505.68±186.49</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>266.7</td>
<td>330.0</td>
<td>514.75</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>190 to 383.7</td>
<td>189.1 to 613.6</td>
<td>159.1 to 984.9</td>
</tr>
<tr>
<td>Critical Time (s)</td>
<td>Mean±SD</td>
<td>8.57±1.11</td>
<td>7.76±0.99</td>
<td>7.05±1.11</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>8.4</td>
<td>7.80</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>7.0 to 11.3</td>
<td>5.7 to 10.0</td>
<td>5.8 to 10.8</td>
</tr>
<tr>
<td>TBPI</td>
<td>Mean±SD</td>
<td>0.998±0.074</td>
<td>0.917±0.153</td>
<td>0.842±0.124</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>1.0</td>
<td>0.960</td>
<td>0.850</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0.8 to 1.2</td>
<td>0.6 to 1.1</td>
<td>0.5 to 1.1</td>
</tr>
<tr>
<td>ABPI</td>
<td>Mean±SD</td>
<td>1.041±0.065</td>
<td>1.027±0.097</td>
<td>1.108±0.142</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>1.04</td>
<td>1.01</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0.94 to 1.20</td>
<td>0.78 to 1.29</td>
<td>0.90 to 1.41</td>
</tr>
</tbody>
</table>
Among the 100 recruited participants, one did not have complete lipid profile data. Hence, the remaining 99 participants were re-grouped into two groups; normolipidaemia (n=29) and dyslipidaemia (n=70) based on the values of lipid biomarkers (total cholesterol, triglyceride, HDL-C and LDL-C) (refer to section 3.1.7). The distribution of the haemorheological parameters in the two groups were again analysed by Shapiro-wilk tests which is shown in the Table 4.3; and descriptive statistics is shown in Table 4.4.

Table 4.3: Distribution of haemorheological parameters in the two groups (normolipidaemia and dyslipidaemia) as assessed by Shapiro-wilk tests

<table>
<thead>
<tr>
<th>Normal distribution (P-value &gt;0.05)</th>
<th>Not normal distribution (P-value &lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{max}}$, $S_{1/2}$</td>
<td>Critical time, critical stress, WBV</td>
</tr>
<tr>
<td></td>
<td>Plasma D-dimer, serum hsCRP,</td>
</tr>
<tr>
<td></td>
<td>Erythrocyte SOD, erythrocyte reduced</td>
</tr>
<tr>
<td></td>
<td>glutathione, urinary isoprostanes,</td>
</tr>
<tr>
<td></td>
<td>ABPI and TBPI</td>
</tr>
</tbody>
</table>

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Table 4.4: Summary of the statistical characteristics of the biomarkers in two groups

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Statistics</th>
<th>Normolipidaemia</th>
<th>Dyslipidaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants (N)</td>
<td></td>
<td>29</td>
<td>70</td>
</tr>
<tr>
<td>Serum hsCRP (mg/L)</td>
<td>Mean±SD</td>
<td>1.97±3.22</td>
<td>2.81±3.07</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>0.70</td>
<td>1.19</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0.10 to 14.20</td>
<td>0.10 to 11.50</td>
</tr>
<tr>
<td>Plasma D-dimer (µg/mL)</td>
<td>Mean±SD</td>
<td>0.50±0.41</td>
<td>0.56±0.66</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>0.39</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0.22 to 2.03</td>
<td>0.22 to 5.39</td>
</tr>
<tr>
<td>Erythrocyte SOD (U/mL)</td>
<td>Mean±SD</td>
<td>581.50±104.70</td>
<td>590.57±93.28</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>528.15</td>
<td>588.94</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>440 to 799</td>
<td>440 to 769</td>
</tr>
<tr>
<td>Erythrocyte GSH (mg/dL)</td>
<td>Mean±SD</td>
<td>57.63±9.66</td>
<td>60.41±8.52</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>59.00</td>
<td>61.55</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>36.0 to 76.0</td>
<td>42.0 to 77.0</td>
</tr>
<tr>
<td>Urinary isoprostanes (ng/mmol)</td>
<td>Mean±SD</td>
<td>102.96±80.54</td>
<td>139.57±94.70</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>98.98</td>
<td>117.88</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>1.01 to 332.07</td>
<td>1.26 to 388.30</td>
</tr>
<tr>
<td>WBV (mPa.s)</td>
<td>Mean±SD</td>
<td>4.22±0.54</td>
<td>4.22±0.44</td>
</tr>
<tr>
<td></td>
<td>Median</td>
<td>4.20</td>
<td>4.15</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>3.20 to 5.80</td>
<td>3.13 to 5.53</td>
</tr>
</tbody>
</table>
Table 4.4: contd....

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Statistics</th>
<th>Normolipidaemia</th>
<th>Dyslipidaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>0.55±0.02</td>
<td>0.54±0.02</td>
</tr>
<tr>
<td><strong>EI&lt;sub&gt;max&lt;/sub&gt;</strong></td>
<td>Median</td>
<td>0.56</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0.50 to 0.61</td>
<td>0.50 to 0.61</td>
</tr>
<tr>
<td></td>
<td>Mean±SD</td>
<td>2.65±0.45</td>
<td>2.79±0.46</td>
</tr>
<tr>
<td><strong>SS&lt;sub&gt;1/2&lt;/sub&gt;</strong></td>
<td>Median</td>
<td>2.54</td>
<td>2.71</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>2.0 to 4.0</td>
<td>2.0 to 4.0</td>
</tr>
<tr>
<td></td>
<td>Mean±SD</td>
<td>316.87±119.03</td>
<td>403.11±168.61</td>
</tr>
<tr>
<td><strong>Critical stress (Pa)</strong></td>
<td>Median</td>
<td>289.70</td>
<td>355.45</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>175.8 to 685.6</td>
<td>159.1 to 984.9</td>
</tr>
<tr>
<td></td>
<td>Mean±SD</td>
<td>8.16±1.15</td>
<td>7.62±1.22</td>
</tr>
<tr>
<td><strong>Critical Time (s)</strong></td>
<td>Median</td>
<td>8.10</td>
<td>7.50</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>5.7 to 11.1</td>
<td>5.8 to 11.3</td>
</tr>
</tbody>
</table>

**Statistical tool selection:** It has been suggested that it is better to use non-parametric tests for non-normal data than using parametric tests after attempting to normalise the data (Leech & Onwuegbuzie, 2002). Hence, parametric tests (mean comparison, Pearson’s correlation) were used for the analysis of normally distributed markers whereas non-parametric tests (median comparisons, Spearman’s correlation) were used for the analysis of non-normal distribution (refer to 3.5).
4.2 Oxidative stress and chronic inflammation in metabolic syndrome

4.2.1 Mean comparison of age among three groups (I, II and III)

Mean age of participants was compared among three groups (I, II and III) (refer to section 3.1.6) using ANOVA tests. The differences in mean age between three groups (45.48±8.974, 56.52±12.94 and 63.06±12.13 years) were significant ($P<0.0005$). Tukey post-hoc analysis revealed that the increase from group I to II ($P=0.001$) and from group I to group III ($P<0.0005$) were significant but the age difference between group II and III was not significant ($P=0.054$).

Since there was a significant difference in the age of participants in three different groups, age was adjusted in all the regression analysis for predicting and associating the MetS with biomarkers.

4.2.2 Median comparison

hsCRP, D-dimer, SOD, GSH and urinary isoprostanes were not normally distributed across the three different groups (Group I, II and III; refer to section 3.1.6) as assessed by Shapiro-Wilk test ($P<0.05$). Hence, non-parametric tests were used for the analysis (Table 4.1). The inter-assay coefficient of variations for glutathione, isoprostanes and SOD were 2.1%, 6.5% and 5.2% respectively.

There were significant differences in the median value of hsCRP, D-dimer, SOD, GSH and urinary isoprostanes across the three different studied groups as shown in Table 4.5.
Table 4.5: Median values of oxidative stress and inflammatory markers in three different groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hs-CRP (mg/L)</td>
<td>0.44</td>
<td>1.25</td>
<td>2.33</td>
<td>0.001</td>
</tr>
<tr>
<td>D-dimer (µg/mL)</td>
<td>0.36</td>
<td>0.39</td>
<td>0.53</td>
<td>0.003</td>
</tr>
<tr>
<td>SOD (U/mL)</td>
<td>630.0</td>
<td>587.6</td>
<td>524.7</td>
<td>0.021</td>
</tr>
<tr>
<td>GSH (mg/dL)</td>
<td>65.1</td>
<td>61.80</td>
<td>52.0</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Urinary isoprostanes</td>
<td>65.18</td>
<td>144.09</td>
<td>165.71</td>
<td>0.001</td>
</tr>
</tbody>
</table>

4.2.3 Post-hoc analysis of median comparison among three groups

Pairwise comparisons were performed using Dunn’s (1964) procedure with a Benferroni correction for multiple comparisons. Post-hoc pairwise comparisons revealed that hsCRP level was significantly different between group II and group III (P=0.015) and between group I and group III (P=0.001) (Table 4.5). There was no significant median difference between group I and II (P=0.371). D-dimer level was significantly different between group I and group III (P=0.003) (Table 4.5). There was no significant difference between group II and III (P=0.736) and between group I and II (P=0.086). SOD level was significantly different between group I and group III (P=0.018) (Table 4.5). There was no significant difference between group II and III (P=0.221) and between group I and II (P=0.349).
GSH level was significantly different between group I and group II \((P=0.001)\) and between group I and group III \((P<0.0005)\) (Table 4.5). There was no significant difference between group II and III \((P=0.297)\). In the case of urinary isoprostanes level, post-hoc pairwise statistics revealed the significant difference between group I and III only \((P=0.001)\) (Table 4.5).

### 4.3 Dyslipidaemia and altered haemorheology

Among the 100 recruited participants, one did not have complete lipid profile data. Hence, the remaining 99 participants were re-grouped into two groups; normolipidaemia \((n=29)\) and dyslipidaemia \((n=70)\) based on the values of lipid biomarkers (total cholesterol, triglyceride, HDL-C and LDL-C). In dyslipidaemic group; 21 had one abnormal lipid parameter, 36 had two abnormal lipid parameters, 11 had three abnormal lipid parameters and 2 had all four abnormal lipid parameters. The difference in mean age \((\pm SD)\) between normolipidaemic and dyslipidaemic group was not significant \((57.66\pm15.39 \text{ Vs } 54.43\pm12.75 \text{ years, } P \text{ value}=0.284)\).

#### 4.3.1 Mean comparison for normally distributed data

\(E_{\text{Imax}}\) and \(SS_{1/2}\) were normally distributed for both normolipidaemic and dyslipidaemic groups \((P>0.05)\) (refer to Table 4.2).

Though the assumption of homogeneity of variance was not violated, as assessed by Levene’s test for equality of variances \((P>0.05)\), Welch t-test was used to compare means of \(E_{\text{Imax}}\) and \(SS_{1/2}\) between normolipidemic and dyslipidemic groups because of large difference in sample size between groups \((n=29 \text{ Vs } 70)\) as recommended by Howell (Howell, 2010). There were no significant differences in mean value of \(E_{\text{Imax}}\) \((0.558\pm0.027 \text{ Vs } 0.546\pm0.026, P=0.060)\) and \(SS_{1/2}\) \((2.65\pm0.45 \text{ Vs } 2.79\pm0.46, P=0.173)\) between normolipidaemic and dyslipidaemic groups.
4.3.2 Median comparison for non-normal data

Critical time, critical stress and WBV were not normally distributed in either or both groups ($P<0.05$) as assessed by Shapiro-Wilk Tests and hence non-parametric tests were used for the analysis (Table 4.2).

A Mann-Whitney U test was used to determine if there were differences in erythrocyte aggregation parameters and WBV between normolipidaemic and dyslipidaemic groups. Distribution of the parameters analysed (critical time/ critical stress and WBV), for both groups were similar, as assessed by visual inspection. Median score of the parameters in two groups along with U and Z values are given in Table 4.6.

Table 4.6: Table showing median values, Mann-whitney U value, Z and $P$ value.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Median Normolipidaemic (n=29)</th>
<th>Median Dyslipidaemic (n=70)</th>
<th>U</th>
<th>Z</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Critical time (s)</td>
<td>8.10</td>
<td>7.5</td>
<td>707.5</td>
<td>-2.3</td>
<td>0.018</td>
</tr>
<tr>
<td>Critical stress (Pa)</td>
<td>289.70</td>
<td>355.45</td>
<td>1370.0</td>
<td>2.7</td>
<td>0.006</td>
</tr>
<tr>
<td>WBV (mPa.s)</td>
<td>4.20</td>
<td>4.15</td>
<td>1009.5</td>
<td>-0.042</td>
<td>0.966</td>
</tr>
</tbody>
</table>
4.3.3 Correlation between haemorheological parameters and lipid parameters

Correlational analysis was done to study the correlation between lipid and haemorheological parameters. WBV was not significantly correlated with any of the lipids risk factors. Erythrocyte aggregation parameters (critical stress and critical time) showed significant correlation with HDL-C and triglyceride. Critical stress also showed negative correlation with LDL-C. Erythrocyte deformability parameters, SS₁/₂ did not show significant correlation with lipid parameters whereas EIₘₐₓ showed significant correlation with HDL-C and triglyceride (Table 4.7).

| Table 4.7: correlation* between lipids and hemorheological parameters |
|-------------------|------------------|----------------|------------------|------------------|------------------|
|                   | HDL-C            | triglyceride   | LDL-C            | non-HDL-C        | TC†              |
| WBV               |                  |                |                  |                  |                  |
| R                 | -.177            | .187           | -.137            | -.081            | -.148            |
| P-Value           | .080             | .064           | .177             | .427             | .145             |
| Critical Time     |                  |                |                  |                  |                  |
| r                 | .280             | -.438          | .097             | -.055            | .034             |
| P-Value           | .005             | <0.0005        | .340             | .591             | .737             |
| Critical Stress   |                  |                |                  |                  |                  |
| R                 | -.459            | .591           | -.204            | .029             | -.117            |
| P-Value           | <0.0005          | <0.0005        | .043             | .777             | .248             |
| EIₘₐₓ             |                  |                |                  |                  |                  |
| R                 | .299             | -.368          | .112             | -.024            | .082             |
| P-Value           | .003             | <0.0005        | .269             | .790             | .421             |
| SS₁/₂             |                  |                |                  |                  |                  |
| R                 | -.171            | .124           | -.119            | -.069            | -.123            |
| P-Value           | .091             | .223           | .240             | .499             | .224             |

*Pearson’s correlation (parametric) for EIₘₐₓ and SS₁/₂ and Spearman’s correlation (non-parametric) for Critical time, critical stress and WBV
†TC: total cholesterol
4.3.4 Ordinal logistic regression analysis

The WBV, $E_{I_{\text{max}}}$ and critical stress were divided into four groups (quartiles) using 25th, 50th and 75th percentiles. The values in the highest quartile for WBV (hyperviscosity) and critical stress (hyperaggregation) were considered abnormal (high) whereas the values at the lowest quartile of $E_{I_{\text{max}}}$ were considered abnormal (low) for the analysis purpose. The haemorheological parameters in different quartiles are shown in Table 4.8.

Table 4.8: Haemorheological parameters in different quartiles

<table>
<thead>
<tr>
<th>Quartile</th>
<th>Critical stress (Pa)</th>
<th>Critical time (s)</th>
<th>WBV (mPa.s)</th>
<th>$E_{I_{\text{max}}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>&lt;259.85</td>
<td>&lt;7.0</td>
<td>&lt;3.9</td>
<td>&lt;0.5250</td>
</tr>
<tr>
<td>Second</td>
<td>259.85-329.25</td>
<td>7.0-7.6</td>
<td>3.9-4.17</td>
<td>0.5250-0.5510</td>
</tr>
<tr>
<td>Third</td>
<td>329.25-476.9</td>
<td>7.6-8.5</td>
<td>4.17-4.43</td>
<td>0.5510-0.5690</td>
</tr>
<tr>
<td>Fourth</td>
<td>$\geq$476.9</td>
<td>$\geq$8.5</td>
<td>$\geq$4.43</td>
<td>$\geq$0.5690</td>
</tr>
</tbody>
</table>

Ordinal Logistic regression (Generalised Linear Model) showed that the dyslipidaemia occurred 2.825 (95% CI, 1.267 to 6.299) times more frequently than normolipidaemia, on the highest quartile of erythrocyte aggregation (critical stress), a significant effect, $\chi^2 (1)=6.445$, $P=0.011$. There was no significant association of dyslipidaemia with the quartiles of WBV ($P>0.05$) and $E_{I_{\text{max}}}$ ($P=0.070$).
4.3.5 ROC curve analysis

ROC curve was constructed and area under the curve (AUC) was determined to confirm the association of lipids parameters with hyperaggregation (Table 4.9). AUC was highest for triglyceride followed by HDL-C for correctly classifying hyperaggregation (critical stress >476.9)

Table 4.9: AUC and 95% CI obtained from ROC curve analysis for classifying hyperaggregation by lipids parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AUC</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride</td>
<td>0.831</td>
<td>0.740 to</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>HDL-C</td>
<td>0.800</td>
<td>0.694 to</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>LDL-C</td>
<td>0.259</td>
<td>0.137 to</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.324</td>
<td>0.196 to</td>
<td>0.010</td>
</tr>
</tbody>
</table>
4.4 Metabolic syndrome and altered haemorheology

4.4.1 Mean comparison of WBV (among three groups)

WBV, critical stress and EI_{max} were normally distributed ($P>0.05$) in all three groups (Table 4.1). The mean difference of WBV in three different groups is shown in Table 4.10.

Table 4.10: ANOVA tests for WBV among three different groups

<table>
<thead>
<tr>
<th>WBV (shear stress)</th>
<th>Group I (mPa.s)</th>
<th>Group II (mPa.s)</th>
<th>Group III (mPa.s)</th>
<th>$P$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBV 45 s$^{-1}$</td>
<td>5.147±0.51</td>
<td>5.42±0.47</td>
<td>5.54±0.47</td>
<td>0.004</td>
</tr>
<tr>
<td>WBV 55 s$^{-1}$</td>
<td>4.99±0.47</td>
<td>5.29±0.42</td>
<td>5.35±0.40</td>
<td>0.002</td>
</tr>
<tr>
<td>WBV 100 s$^{-1}$</td>
<td>4.25±0.34</td>
<td>4.70±0.50</td>
<td>4.90±0.39</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>WBV 150 s$^{-1}$</td>
<td>3.99±0.33</td>
<td>4.10±0.50</td>
<td>4.56±0.36</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>WBV 200 s$^{-1}$</td>
<td>3.79±0.22</td>
<td>4.10±0.43</td>
<td>4.47±0.37</td>
<td>&lt;0.0005</td>
</tr>
</tbody>
</table>

4.4.2 Post-hoc analysis of ANOVA test for WBV

At the shear rate of 150 s$^{-1}$, mean WBV value increased from group I (3.99±0.33) to group II (4.10±0.50) and group III (4.56±0.36). Tukey post-hoc analysis revealed that the difference between group I and III ($P<0.0005$) and II and III ($P=0.042$) were significant (Table 4.10).

4.4.3 Mean comparison of critical stress (among three groups)

A one way ANOVA was conducted to determine if critical stress was different for three different groups. Critical stress values were significantly different among the three groups, Welch’s F(2, 55.481)=28.96, $P<0.0005$ (Table 4.11).
Table 4.11: ANOVA test for Critical Stress among three groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Critical stress (Welch) (Pa)</td>
<td>274.23±48.44</td>
<td>343.48±101.34</td>
<td>505.68±186.49</td>
<td>&lt;0.0005</td>
</tr>
</tbody>
</table>

4.4.4 Post-hoc analysis of ANOVA test for critical stress

Critical stress mean value increased from group I (274.23±48.44 Pa) to the group II (343.48±101.34 Pa) and group III (505.68±186.49 Pa). Post-hoc analysis revealed that the increase in mean value from group I to group II (69.24, 95% CI: 21.62 to 116.86, \( P=0.003 \)) and from group II to group III (162.20, 95% CI: 76.11 to 248.29, \( P<0.0005 \)) was significant (Table 4.11).

4.4.5 Mean comparison of \( E_{I_{\text{max}}} \) (among three groups)

A one way ANOVA was used to determine if mean value of \( E_{I_{\text{max}}} \) and \( E_{I@3} \) Pa was different for three different groups. \( E_{I_{\text{max}}} \) and \( E_{I@3} \) Pa values were found to be significantly different between three groups (Table 4.12).

Table 4.12: ANOVA test for \( E_{I_{\text{max}}} \) and \( E_{I@3} \) Pa among three groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E_{I_{\text{max}}} )</td>
<td>0.561±0.02</td>
<td>0.557±0.022</td>
<td>0.534±0.214</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>( E_{I@3} ) Pa</td>
<td>0.311±0.035</td>
<td>0.304±0.019</td>
<td>0.293±0.029</td>
<td>0.043</td>
</tr>
</tbody>
</table>
4.4.6 Post-hoc analysis of ANOVA test for $\text{EI}_{\text{max}}$

Mean $\text{EI}_{\text{max}}$ values decreased from group I (0.561±0.02) to the group II (0.557±0.022) and further decreased in the group III (0.534±0.214). Tukey post-hoc analysis revealed that the decrease from group II to III ($P=0.001$) and I to III ($P<0.0005$) was significant but decrease from group I to group II was non-significant ($P=0.766$) (Table 4.12).

![Figure 4.1](image)

**Figure 4.1** Box-plot showing the mean values of WBV, critical stress and $\text{EI}_{\text{max}}$ on three different groups.
4.4.7 Median comparison of critical time and $SS_{1/2}$ (non-normal distribution) among three groups

Since the distribution of critical time, and $SS_{1/2}$ were not normal (Table 4.1), Kruskal-Wallis test was used to compare the median between three groups. There was a significant difference in the median value of critical time between three groups (Table 4.13) but no significant median difference was seen in the level of $SS_{1/2}$.

Table 4.13: Median and $P$-value for Kruskal-Wallis test

<table>
<thead>
<tr>
<th>Variables</th>
<th>Median</th>
<th>Test Statistic</th>
<th>$P$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group I</td>
<td>Group II</td>
<td>Group III</td>
</tr>
<tr>
<td>Critical time (s)</td>
<td>8.4</td>
<td>7.8</td>
<td>7.0</td>
</tr>
<tr>
<td>$SS_{1/2}$</td>
<td>2.60</td>
<td>2.55</td>
<td>2.90</td>
</tr>
</tbody>
</table>

4.4.8 Post-hoc analysis of Kruskal-Wallis test for critical time

For the variables with significant Kruskal-Wallis test (critical time), pairwise post-hoc test was performed (Table 4.14). The pairwise comparison was performed using Dunn’s (1964) procedure with a Benferroni correction for multiple comparisons. The alpha level was reduced with a new adjusted $P$-value=$0.05/3=0.016$. 
4.4.9 Correlational analysis of haemorheological parameters with MetS components

Spearman’s Correlation was performed to correlate haemorheological parameters with the components of MetS. Critical time and critical stress correlated with all of the five components of MetS (Table 4.15) suggesting that erythrocyte aggregation was significantly correlated with MetS. Except for systolic blood pressure, $E_{\text{I max}}$ was significantly correlated with all of the components of MetS. $SS_{1/2}$ was significantly negatively correlated only with HDL-C. In the same way, $WBV$ was significantly correlated with systolic blood pressure, waist circumference an $FBG$ (Table 4.15).
Table 4.15: Correlation* of haemorheological parameters with the components of MetS

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Systolic blood pressure</th>
<th>Waist circumference</th>
<th>HDL-C</th>
<th>Triglyceride</th>
<th>FBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBV</td>
<td>Correlation Coefficient</td>
<td>.264</td>
<td>.344</td>
<td>-.177</td>
<td>.187</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>.008</td>
<td>.000</td>
<td>.080</td>
<td>.064</td>
</tr>
<tr>
<td>Crit Stress</td>
<td>Correlation Coefficient</td>
<td>.383</td>
<td>.419</td>
<td>-.459</td>
<td>.591</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>.000</td>
<td>.000</td>
<td>.000</td>
<td>.000</td>
</tr>
<tr>
<td>Crit time</td>
<td>Correlation Coefficient</td>
<td>-.426</td>
<td>-.449</td>
<td>.280</td>
<td>-.438</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>.000</td>
<td>.000</td>
<td>.005</td>
<td>.000</td>
</tr>
<tr>
<td>Elmax</td>
<td>Correlation Coefficient</td>
<td>-.150</td>
<td>-.304</td>
<td>.362</td>
<td>-.388</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>.136</td>
<td>.002</td>
<td>.000</td>
<td>.000</td>
</tr>
<tr>
<td>SS1/2</td>
<td>Correlation Coefficient</td>
<td>-.067</td>
<td>.039</td>
<td>-.255</td>
<td>.143</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>.508</td>
<td>.698</td>
<td>.011</td>
<td>.157</td>
</tr>
</tbody>
</table>

*Spearman’s correlation was used because correlating parameters (one or both) do not follow normal distribution.

4.4.10 Binomial logistic Regression Analysis

The WBV, Elmax and critical stress was divided into four groups (quartiles) using 25th, 50th and 75th percentiles. The values in the highest quartile for WBV (hyperviscosity) and critical stress (hyperaggregation) were considered abnormal (high) whereas the values at the lowest quartile of Elmax were considered abnormal (low) for the analysis. The haemorheological parameters in different quartiles are given in Table 4.9.
Binomial logistic regression analysis was run to predict hyperviscosity (WBV at highest quartile), erythrocyte hyperaggregation (critical stress at highest quartile) and low erythrocyte deformability (EImax at lowest quartile) (Tables 4.16, 4.17 and 4.18).

Table 4.16: Prediction of low erythrocyte deformability by MetS components

<table>
<thead>
<tr>
<th>MetS Components</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure</td>
<td>1.022</td>
<td>0.998 to 1.046</td>
<td>0.068</td>
</tr>
<tr>
<td>FBG</td>
<td>1.119</td>
<td>0.913 to 1.371</td>
<td>0.278</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.038</td>
<td>0.997 to 1.082</td>
<td>0.070</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1.004</td>
<td>0.997 to 1.010</td>
<td>0.263</td>
</tr>
<tr>
<td>Waist Circumference</td>
<td>1.021</td>
<td>0.995 to 1.047</td>
<td>0.110</td>
</tr>
</tbody>
</table>

Table 4.17: Prediction of erythrocyte hyperaggregability by MetS components

<table>
<thead>
<tr>
<th>MetS Components</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure</td>
<td>1.055</td>
<td>1.025 to 1.085</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FBG</td>
<td>1.367</td>
<td>1.047 to 1.786</td>
<td>0.022</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.121</td>
<td>1.052 to 1.194</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1.020</td>
<td>1.011 to 1.030</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Waist Circumference</td>
<td>1.052</td>
<td>1.022 to 1.084</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Table 4.18: Prediction of hyperviscosity by MetS components

<table>
<thead>
<tr>
<th>MetS Components</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure</td>
<td>1.018</td>
<td>0.991 to 1.047</td>
<td>0.182</td>
</tr>
<tr>
<td>FBG</td>
<td>1.005</td>
<td>0.980 to 1.032</td>
<td>0.676</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.035</td>
<td>0.994 to 1.077</td>
<td>0.091</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1.004</td>
<td>0.998 to 1.011</td>
<td>0.216</td>
</tr>
<tr>
<td>Waist Circumference</td>
<td>1.005</td>
<td>0.980 to 1.032</td>
<td>0.676</td>
</tr>
</tbody>
</table>

4.4.11 Ordinal logistic regression analysis

Ordinal logistic regression analysis was run through Generalised Linear Model to find out the association of MetS (group III) with highest quartile of WBV and critical stress and lowest quartile of EI\textsubscript{max}. Ordinal logistic regression analysis showed that highest quartile of WBV values occurred 7.958 (95% CI, 2.556 to 24.775) times more frequently than lowest quartile values in participants with MetS, a significant effect, \( \chi^2 (1)=12.816; P<0.0005 \). Similarly, the highest quartile of critical stress values occurred 44.293 (95% CI, 10.680 to 183.696) times more frequently than lowest quartile values in participants with MetS, a significant effect, \( \chi^2 (1)=27.283; P<0.0005 \). In the same way, the lowest quartile of EI\textsubscript{max} values occurred 6.795 (95% CI, 2.244 to 20.573) times more frequently than lowest quartile values in participants with MetS, a significant effect, \( \chi^2 (1)=11.494; P=0.001 \).
4.5 Oxidative stress, inflammation and altered haemorheology

4.5.1 Correlational analysis

Haemorheological parameters were correlated with oxidative stress and inflammatory markers as shown in Table 4.19. Erythrocyte aggregation parameters were significantly correlated with all of the parameters analysed (positively with the level of hsCRP, D-dimer and urinary isoprostanes and negatively with the level of SOD and GSH). \( E_{\text{max}} \) was significantly and negatively correlated only with urinary isoprostanes level whereas \( S_{1/2} \) significantly positively correlated only with SOD level. WBV negatively correlated with GSH and positively with urinary isoprostanes.

Table 4.19: Spearman’s Correlation of haemorheological parameters with inflammatory and oxidative stress markers

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Crit time (s)</th>
<th>Crit Stress (Pa)</th>
<th>( E_{\text{max}} )</th>
<th>( S_{1/2} )</th>
<th>WBV (mPa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsCRP (mg/L)</td>
<td>r</td>
<td>-.517</td>
<td>.450</td>
<td>-.126</td>
<td>.039</td>
</tr>
<tr>
<td>D-dimer (µg/mL)</td>
<td>r</td>
<td>-.262</td>
<td>.314</td>
<td>.011</td>
<td>-.175</td>
</tr>
<tr>
<td>SOD (U/mL)</td>
<td>r</td>
<td>.241</td>
<td>-.198</td>
<td>-.075</td>
<td>.240</td>
</tr>
<tr>
<td>GSH (mg/dL)</td>
<td>r</td>
<td>.336</td>
<td>-.234</td>
<td>.167</td>
<td>.018</td>
</tr>
<tr>
<td>Urinary isoprostanes (ng/mmol)</td>
<td>r</td>
<td>-.288</td>
<td>.257</td>
<td>-.280</td>
<td>.105</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameters</th>
<th>P-Value</th>
<th>P-Value</th>
<th>P-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsCRP (mg/L)</td>
<td>.000</td>
<td>.000</td>
<td>.211</td>
<td>.701</td>
</tr>
<tr>
<td>D-dimer (µg/mL)</td>
<td>.009</td>
<td>.002</td>
<td>.914</td>
<td>.083</td>
</tr>
<tr>
<td>SOD (U/mL)</td>
<td>.016</td>
<td>.048</td>
<td>.458</td>
<td>.016</td>
</tr>
<tr>
<td>GSH (mg/dL)</td>
<td>.001</td>
<td>.019</td>
<td>.097</td>
<td>.860</td>
</tr>
</tbody>
</table>

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4.5.2 Linear regression analysis

To investigate the association between haemorheological parameters and oxidative stress/inflammation, linear regression analysis was used and the results of regression analyses are shown in Table 4.20. Critical time and critical stress were significantly predicted by the level of hsCRP, SOD, GSH and urinary isoprostanes. Urinary isoprostanes and GSH level significantly predicted WBV. $E_{\text{I}_{\text{max}}}$ was significantly predicted by urinary isoprostanes level whereas $SS_{1/2}$ was significantly predicted by SOD level.

<table>
<thead>
<tr>
<th>Predictors (continuous variable)</th>
<th>WBV</th>
<th>$E_{\text{I}_{\text{max}}}$</th>
<th>$SS_{1/2}$</th>
<th>Critical Stress</th>
<th>Critical time</th>
</tr>
</thead>
<tbody>
<tr>
<td>hs-CRP (mg/L)</td>
<td>0.682*</td>
<td>0.110</td>
<td>0.381</td>
<td>&lt;0.0005</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>D-dimer (µg/mL)</td>
<td>0.577</td>
<td>0.943</td>
<td>0.201</td>
<td>0.226</td>
<td>0.358</td>
</tr>
<tr>
<td>SOD (U/mL)</td>
<td>0.273</td>
<td>0.720</td>
<td>0.016</td>
<td>0.078</td>
<td>0.009</td>
</tr>
<tr>
<td>GSH (mg/dL)</td>
<td>0.043</td>
<td>0.106</td>
<td>0.516</td>
<td>0.003</td>
<td>0.004</td>
</tr>
<tr>
<td>Urinary isoprostanes (ng/mmol)</td>
<td>0.005</td>
<td>0.003</td>
<td>0.354</td>
<td>0.018</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* $P$-value of regression analysis given in the table
4.5.3 Trend analysis

Jonckheere Trend test was used to analyse the trend of oxidative stress and inflammatory markers across the quartiles of critical stress (Figure 4.2). There was a linear increase in the level of hsCRP ($P$ trend $<0.0005$), D-dimer ($P$ trend $=0.002$) and urinary isoprostanes ($P$ trend $=0.007$) and a linear decrease in the level of SOD ($P$ trend $=0.035$) and GSH ($P$ trend $=0.004$) across the quartiles of critical stress. On contrary, there was a linear decrease in the level of hsCRP ($P$ trend $<0.0005$), D-dimer ($P$ trend $=0.021$) and urinary isoprostanes ($P$ trend $=0.016$) and a linear increase in the level of SOD ($P$ trend $=0.036$) and GSH ($P$ trend $=0.001$) across the quartiles of critical time. Similarly, D-dimer ($P$ trend $=0.049$) and urinary isoprostanes ($P$ trend $=0.015$) level showed a linear increase and GSH level showed a linear decrease ($P$ trend $=0.005$) across the quartiles of WBV whereas the level of hsCRP ($P$ trend $=0.147$) and SOD ($P$ trend $=0.542$) did not follow any significant trend across the quartiles. Among the markers analysed, only urinary isoprostanes level showed a significant decreasing trend across the quartiles of $EI_{\text{max}}$ ($P$ trend $=0.005$).
Figure 4.2 Scatter plot showing the trend of urinary isoprostanes, GSH and hsCRP across the quartiles of critical stress and WBV.
4.6 Erythrocyte morphology, oxidative stress, chronic inflammation and metabolic syndrome

4.6.1 Mean comparison for the percentage of morphologic subclass among three groups

The mean percentage of different erythrocyte morphology in groups I, II and III is shown in Table 4.21. The mean percentages of biconcave cells were higher in group I when compared to groups II and III. Acanthocytes, echinocytes and stomatocytes were the abnormal cells that were found to be increased in group III.

Table 4.21: Erythrocyte morphology (SEM) (means ±SD) for the percentage of morphological subclasses in three study groups

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Group I (%)</th>
<th>Group II (%)</th>
<th>Group III (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biconcave cells</td>
<td>89.60±2.16</td>
<td>86.97±3.11</td>
<td>84.04±2.95</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Acanthocytes</td>
<td>3.05±1.01</td>
<td>4.48±2.06</td>
<td>6.61±2.18</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Echinocytes</td>
<td>1.02±0.46</td>
<td>1.13±0.62</td>
<td>1.50±0.75</td>
<td>0.006</td>
</tr>
<tr>
<td>Leptocytes</td>
<td>2.66±0.98</td>
<td>3.07±1.15</td>
<td>2.94±1.18</td>
<td>0.335</td>
</tr>
<tr>
<td>Stomatocytes</td>
<td>2.00±0.80</td>
<td>2.60±1.32</td>
<td>3.20±1.39</td>
<td>0.001</td>
</tr>
</tbody>
</table>

4.6.2 Post-hoc analysis of ANOVA tests for erythrocyte morphological subclass

Post-hoc analyses revealed that mean percentage of biconcave cells ($P<0.0005$), acanthocytes ($P<0.0005$), echinocytes ($P=0.007$) and stomatocytes ($P<0.0005$) significantly differed between group I and III (Table 4.21). Similarly, the mean percentage of biconcave cells ($P=0.001$) and acanthocytes ($P=0.007$) significantly differed between group I and II. In the same way, the mean
percentage of biconcave cells \((P<0.0005)\), acanthocytes \((P<0.0005)\) and echinocytes \((P=0.048)\) significantly differed between group II and III.

4.6.3 Correlation between morphologic subclass and other haemorheological parameters

The correlation of morphologically abnormal cells with other haemorheological parameters is shown in Table 4.22. Acanthocytes and echinocytes were positively correlated whereas biconcave cells were negatively correlated with erythrocyte aggregation. Acanthocytes were also negatively correlated with maximum erythrocyte deformability whereas biconcave cells were positively correlated with maximum erythrocyte deformability (Figure 4.3).

Table 4.22: The correlation* of abnormal erythrocyte morphology with erythrocyte deformability and erythrocyte aggregation

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Critical time</th>
<th>Critical Stress</th>
<th>(E_{\text{max}})</th>
<th>(S_{1/2})</th>
<th>WBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthocytes</td>
<td>(r)</td>
<td>-.296</td>
<td>.412</td>
<td>-.332</td>
<td>.167</td>
</tr>
<tr>
<td></td>
<td>(P)</td>
<td>.003</td>
<td>.000</td>
<td>.001</td>
<td>.096</td>
</tr>
<tr>
<td>Echinocytes</td>
<td>(r)</td>
<td>-.149</td>
<td>.270</td>
<td>-.153</td>
<td>.039</td>
</tr>
<tr>
<td></td>
<td>(P)</td>
<td>.139</td>
<td>.007</td>
<td>.130</td>
<td>.701</td>
</tr>
<tr>
<td>Stomatocytes</td>
<td>(r)</td>
<td>-.182</td>
<td>.178</td>
<td>-.127</td>
<td>.168</td>
</tr>
<tr>
<td></td>
<td>(P)</td>
<td>.070</td>
<td>.076</td>
<td>.209</td>
<td>.095</td>
</tr>
<tr>
<td>Leptocytes</td>
<td>(r)</td>
<td>-.077</td>
<td>.033</td>
<td>-.081</td>
<td>-.008</td>
</tr>
<tr>
<td></td>
<td>(P)</td>
<td>.445</td>
<td>.741</td>
<td>.421</td>
<td>.935</td>
</tr>
<tr>
<td>Biconcave</td>
<td>(r)</td>
<td>.336</td>
<td>-.407</td>
<td>.355</td>
<td>-.201</td>
</tr>
<tr>
<td>cells</td>
<td>(P)</td>
<td>.001</td>
<td>.000</td>
<td>.000</td>
<td>.044</td>
</tr>
</tbody>
</table>

*Pearson’s correlation
4.6.4 Correlation between morphologic subclass and oxidative stress/inflammation markers

Morphologically abnormal erythrocytes were significantly correlated with oxidative stress and chronic inflammation markers (Table 4.23). The percentage of normal biconcave cells was negatively correlated with oxidative stress and chronic inflammation. Acanthocytes (%) showed a positive correlation with urinary isoprostanes and hsCRP level and a negative correlation with GSH level. Similarly, stomatocytes (%) was positively correlated with urinary isoprostanes level.
Table 4.23: The correlation* of abnormal erythrocyte morphology with oxidative stress and chronic inflammation parameters

<table>
<thead>
<tr>
<th>morphology</th>
<th>Urinary isoprostanes (ng/mmol)</th>
<th>SOD (U/mL)</th>
<th>GSH (mg/dL)</th>
<th>hsCRP (mg/L)</th>
<th>D-dimer (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthocytes</td>
<td>r</td>
<td>0.623</td>
<td>-0.006</td>
<td>-0.34</td>
<td>0.277</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>&lt;0.005</td>
<td>0.956</td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>Echinocytes</td>
<td>r</td>
<td>-0.039</td>
<td>-0.169</td>
<td>-0.172</td>
<td>0.183</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.703</td>
<td>0.094</td>
<td>0.087</td>
<td>0.069</td>
</tr>
<tr>
<td>Stomatocytes</td>
<td>r</td>
<td>0.341</td>
<td>-0.1</td>
<td>-0.153</td>
<td>0.164</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.001</td>
<td>0.325</td>
<td>0.128</td>
<td>0.103</td>
</tr>
<tr>
<td>Leptocytes</td>
<td>r</td>
<td>0.144</td>
<td>0.018</td>
<td>0.02</td>
<td>-0.073</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.152</td>
<td>0.86</td>
<td>0.841</td>
<td>0.472</td>
</tr>
<tr>
<td>Biconcave cells</td>
<td>r</td>
<td>-0.585</td>
<td>0.09</td>
<td>0.282</td>
<td>-0.292</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>&lt;0.005</td>
<td>0.375</td>
<td>0.005</td>
<td>0.003</td>
</tr>
</tbody>
</table>

*Pearson’s correlation
4.7 Peripheral vascular diseases markers: ABPI and TBPI and its association with haemorheology, oxidative stress and chronic inflammation

4.7.1 Median comparison

The distribution of ABPI and TBPI were not normal as assessed by Shapiro-wilk test ($P<0.05$), and therefore, Kruskal-Wallis test was used to compare the median between three groups (Table 4.1). There was a significant difference in the median value of ABPI and TBPI among three groups (Table 4.24).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Median</th>
<th>Test Statistic</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group I</td>
<td>Group II</td>
<td>Group III</td>
</tr>
<tr>
<td>ABPI</td>
<td>1.04</td>
<td>1.01</td>
<td>1.06</td>
</tr>
<tr>
<td>TBPI</td>
<td>1.0</td>
<td>0.96</td>
<td>0.85</td>
</tr>
</tbody>
</table>

4.7.2 Post-hoc analysis of Kruskal-Wallis test for ABPI and TBPI

The pairwise comparison was performed using Dunn’s (1964) procedure with a Benferroni correction for multiple comparisons. The alpha level was reduced with a new adjusted $P$-value=$0.05/3=0.016$. Post-hoc analysis revealed that there was a significant difference in the median value of TBPI between groups I and III and groups II and III (Table 4.25). Similarly, for ABPI, the significant difference was found only between groups II and III (Table 4.25).
Table 4.25: Kruskal-Wallis post-hoc analysis (pairwise comparisons) with Benferroni corrections

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
<th>P-Value</th>
<th>Remarks*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABPI</td>
<td>Groups I and II</td>
<td>0.579</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Groups I and III</td>
<td>0.039</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Groups II and III</td>
<td>0.008</td>
<td>Significant</td>
</tr>
<tr>
<td>TBPI</td>
<td>Groups I and II</td>
<td>0.029</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Groups I and III</td>
<td>&lt;0.0005</td>
<td>Significant</td>
</tr>
<tr>
<td></td>
<td>Groups II and III</td>
<td>0.005</td>
<td>Significant</td>
</tr>
</tbody>
</table>

4.7.3 Correlation analysis

Spearman’s correlation was performed to correlate ABPI/TBPI with the components of MetS and haemorheological parameters. TBPI showed negative significant correlation with brachial systolic blood pressure, waist circumference and fasting glucose level suggesting that as the values of these components increases, toe pressure decreases (Table 4.26). TBPI also significantly correlated with most of the haemorheological parameters except SS_{1/2} and EI_{max} whereas ABPI showed significant correlation only with WBV (Table 4.27).
Table 4.26: Correlation* of TBPI and ABPI with the components of MetS

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Systolic blood pressure</th>
<th>Waist circumference</th>
<th>HDL-C</th>
<th>Triglyceride</th>
<th>FBG</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABPI</td>
<td>Correlation Coefficient</td>
<td>-.023</td>
<td>.173</td>
<td>-.240</td>
<td>.073</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>.820</td>
<td>.085</td>
<td>.017</td>
<td>.473</td>
</tr>
<tr>
<td>TBPI</td>
<td>Correlation Coefficient</td>
<td>-.545</td>
<td>-.462</td>
<td>.138</td>
<td>-.113</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>&lt;0.0005</td>
<td>&lt;0.0005</td>
<td>.173</td>
<td>.265</td>
</tr>
</tbody>
</table>

*Spearman’s correlation

Table 4.27: Correlation* of haemorheological parameters with ABPI and TBPI

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WBV</th>
<th>Critical Stress</th>
<th>Critical time</th>
<th>EI_{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABPI</td>
<td>.219</td>
<td>.160</td>
<td>-.068</td>
<td>-.180</td>
</tr>
<tr>
<td></td>
<td>.029</td>
<td>.112</td>
<td>.503</td>
<td>.072</td>
</tr>
</tbody>
</table>

* Spearman’s correlation

The median value of TBPI showed a positive correlation with the GSH and SOD level and showed a negative correlation with the hsCRP and D-dimer level. On the other hand, ABPI showed a negative correlation only with the GSH level (Table 4.28).
Table 4.28: Spearman’s correlation* between ABPI and TBPI with oxidative stress/inflammatory markers

<table>
<thead>
<tr>
<th>Parameters</th>
<th>GSH</th>
<th>Urinary isoprostanes</th>
<th>SOD</th>
<th>hsCRP</th>
<th>D-dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABPI</td>
<td>( r = -0.267 )</td>
<td>( r = -0.041 )</td>
<td>( r = -0.123 )</td>
<td>( r = -0.013 )</td>
<td>( r = 0.121 )</td>
</tr>
<tr>
<td></td>
<td>( P = 0.007 )</td>
<td>( P = 0.685 )</td>
<td>( P = 0.222 )</td>
<td>( P = 0.897 )</td>
<td>( P = 0.234 )</td>
</tr>
<tr>
<td>TBPI</td>
<td>( r = 0.424 )</td>
<td>( r = -0.187 )</td>
<td>( r = 0.260 )</td>
<td>( r = -0.156 )</td>
<td>( r = -0.262 )</td>
</tr>
<tr>
<td></td>
<td>( P &lt; 0.0005 )</td>
<td>( P = 0.063 )</td>
<td>( P = 0.009 )</td>
<td>( P = 0.121 )</td>
<td>( P = 0.009 )</td>
</tr>
</tbody>
</table>

4.7.4 Regression analysis

Linear regression analysis was performed to find out the association between peripheral vascular disease markers and oxidative stress/chronic inflammation (Table 4.29). TBPI was significantly predicted by all of the oxidative stress markers but not by hsCRP, whereas ABPI was significantly predicted only by GSH.

Table 4.29: Table showing the \( P \)-value for the linear regression analysis for prediction of ABPI and TBPI by inflammatory and oxidative stress markers

<table>
<thead>
<tr>
<th>Predictors (continuous variables)</th>
<th>Outcome variables</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ABPI</td>
</tr>
<tr>
<td>GSH (mg/dL)</td>
<td>0.031*</td>
</tr>
<tr>
<td>SOD (U/mL)</td>
<td>0.564</td>
</tr>
<tr>
<td>Urinary isoprostanes (ng/mmol)</td>
<td>0.667</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>0.238</td>
</tr>
<tr>
<td>D-dimer (µg/mL)</td>
<td>0.216</td>
</tr>
</tbody>
</table>

\*\( P \)-value of regression analysis given in the table
4.7.5 Trend analysis

Jonckheere Trend test was used to analyse the trend of ABPI and TBPI across the quartiles of oxidative stress and inflammatory markers (Figure 4.4). There was a significant linear increase in the level of TBPI across the quartiles of GSH ($P_{\text{trend}} < 0.0005$) and SOD ($P_{\text{trend}} = 0.009$) and a significant linear decrease across the quartile of D-dimer ($P_{\text{trend}} = 0.007$). Similarly, ABPI showed a significant linear decrease across the quartiles of GSH ($P_{\text{trend}} = 0.012$) (Figure 4.4). ABPI and TBPI did not show significant patterns across the quartiles of urinary isoprostanes and hsCRP.

**Figure 4.4** Scatter plot showing the trend of ABPI and TBPI across the quartiles of GSH level.
4.7.6 Association of TBPI with haemorheological parameters: Regression analysis

Lowest quartile of TBPI (0.48 to 0.83) was significantly predicted by critical stress and WBV (Table 4.30).

Table 4.30: Binomial logistic regression analysis for prediction of lowest quartile TBPI by haemorheological parameters

<table>
<thead>
<tr>
<th>Predictors</th>
<th>TBPI lowest quartile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Critical stress (Pa)</td>
<td>0.025</td>
</tr>
<tr>
<td>Critical time (s)</td>
<td>0.078</td>
</tr>
<tr>
<td>WBV (mPa s)</td>
<td>0.002</td>
</tr>
<tr>
<td>EI_{max}</td>
<td>0.576</td>
</tr>
</tbody>
</table>

*P*-value of regression analysis given in the table

4.7.7 Trend of haemorheological parameters across the quartiles of TBPI

Jonckheere Trend test was used to analyse the trend of haemorheological parameters across the quartiles of TBPI. There was a significant linear decrease in the level of critical stress (*P* trend 0.005) and WBV (*P* trend <0.0005) and significant linear increase in the level of critical time (*P* trend 0.009) across the quartiles of TBPI (Table 4.31).
Table 4.31: Median values of haemorheological parameters across the quartiles of TBPI

<table>
<thead>
<tr>
<th>TBPI Quartile</th>
<th>Critical time</th>
<th>Critical Stress</th>
<th>WBV 150 s(^{-1})</th>
<th>(E_{\text{Imax}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>7.300</td>
<td>429.750</td>
<td>4.3850</td>
<td>.54900</td>
</tr>
<tr>
<td>Second</td>
<td>7.500</td>
<td>356.900</td>
<td>4.1800</td>
<td>.55100</td>
</tr>
<tr>
<td>Third</td>
<td>7.800</td>
<td>340.000</td>
<td>4.2000</td>
<td>.56500</td>
</tr>
<tr>
<td>Last</td>
<td>8.050</td>
<td>286.800</td>
<td>4.0550</td>
<td>.54150</td>
</tr>
</tbody>
</table>

4.8 Association of altered haemorheology, peripheral vascular diseases markers and novel cardiovascular risk factors with metabolic syndrome

4.8.1 Binomial logistic regression analysis

Binomial logistic regression analysis (adjusted for age and sex) was performed to predict the chances of having MetS by altered haemorheological parameters; novel CVDs risk factors and peripheral vascular diseases markers (Table 4.32). All of the markers were divided into quartiles and the odds of having MetS after increase or decrease \((E_{\text{Imax}}, \text{TBPI})\) in one quartile of the markers was estimated. The results show that all of the markers significantly predicted MetS and the Odds ratio was highest for erythrocyte aggregation followed by erythrocyte deformability. When the analysis was performed after adjusting for the effect of age, sex and presence of DM, TBPI and D-dimer did not significantly predicted MetS whereas other markers still showed significant association (Table 4.33).
Table 4.32: Age and sex adjusted odds ratio for predicting MetS by haemorheological parameters, ABPI and TBPI.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Critical stress (quartile)</td>
<td>3.896</td>
<td>2.174 to 6.985</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>EI_{max} (quartile)</td>
<td>2.840</td>
<td>1.666 to 4.830</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>WBV (quartile)</td>
<td>1.823</td>
<td>1.030 to 1.114</td>
<td>0.009</td>
</tr>
<tr>
<td>TBPI (quartile)</td>
<td>1.828</td>
<td>1.059 to 3.154</td>
<td>0.030</td>
</tr>
<tr>
<td>Urinary isoprostanes (quartile)</td>
<td>1.715</td>
<td>1.096 to 2.683</td>
<td>0.018</td>
</tr>
<tr>
<td>hsCRP (quartile)</td>
<td>2.090</td>
<td>1.297 to 3.370</td>
<td>0.002</td>
</tr>
<tr>
<td>D-dimer (quartile)</td>
<td>1.639</td>
<td>1.035 to 2.595</td>
<td>0.035</td>
</tr>
</tbody>
</table>

Table 4.33: Age, sex and diabetes adjusted odds ratio for predicting MetS by haemorheological parameters, ABPI and TBPI

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Critical stress (quartile)</td>
<td>3.770</td>
<td>2.092 to 6.793</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>EI_{max} (quartile)</td>
<td>2.994</td>
<td>1.721 to 5.235</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>WBV (quartile)</td>
<td>1.741</td>
<td>1.098 to 2.760</td>
<td>0.018</td>
</tr>
<tr>
<td>TBPI (quartile)</td>
<td>1.647</td>
<td>0.934 to 2.906</td>
<td>0.084</td>
</tr>
<tr>
<td>Urinary isoprostanes (quartile)</td>
<td>1.776</td>
<td>1.117 to 2.824</td>
<td>0.015</td>
</tr>
<tr>
<td>hsCRP (quartile)</td>
<td>2.185</td>
<td>1.325 to 3.603</td>
<td>0.002</td>
</tr>
<tr>
<td>D-dimer (quartile)</td>
<td>1.551</td>
<td>0.970 to 2.480</td>
<td>0.067</td>
</tr>
</tbody>
</table>
4.8.2 ROC Curve analysis

To confirm the above analysis, the ROC curve of the markers was plotted for the differentiation of MetS and non-MetS at various coordinate points (Figure 4.5). Area under the curve was higher for the haemorheological parameters (erythrocyte aggregation and erythrocyte deformability) than for the TBPI or other novel cardiovascular risk factors (Table 4.34).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AUC</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Critical stress</td>
<td>0.818</td>
<td>0.715 to 0.922</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>EI&lt;sub&gt;max&lt;/sub&gt;</td>
<td>0.782</td>
<td>0.688 to 0.876</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>TBPI</td>
<td>0.774</td>
<td>0.679 to 0.869</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>WBV</td>
<td>0.719</td>
<td>0.616 to 0.821</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Urinary</td>
<td>0.706</td>
<td>0.603 to 0.809</td>
<td>0.001</td>
</tr>
<tr>
<td>D-dimer</td>
<td>0.695</td>
<td>0.583 to 0.807</td>
<td>0.001</td>
</tr>
<tr>
<td>hsCRP</td>
<td>0.661</td>
<td>0.549 to 0.774</td>
<td>0.008</td>
</tr>
</tbody>
</table>
Figure 4.5 ROC curve for haemorheological parameters, novel cardiovascular risk factors and peripheral vascular diseases markers for correctly classifying MetS
4.8.3 Biochemical markers and the severity of the disease

Some of the markers analysed were not significantly different between group II and group III. This suggests that these markers were not sensitive enough to differentiate the severity of the metabolic diseases (one or two positive components versus three or more positive components). However, some markers as shown in the table X showed a significant difference between group II and group III. This indicates that the changes in the concentration of these markers were sensitive enough to differentiate the severity of the metabolic alterations.

Table 4.35: Table summarising the markers that were significantly different between group II and group III (with one or two positive components Vs three or more positive components)

<table>
<thead>
<tr>
<th>Markers</th>
<th>Group II</th>
<th>Group III</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsCRP</td>
<td>1.25</td>
<td>2.33</td>
<td>0.015</td>
</tr>
<tr>
<td>WBV (150s⁻¹)</td>
<td>4.10±0.50</td>
<td>4.56±0.36</td>
<td>0.042</td>
</tr>
<tr>
<td>Critical stress</td>
<td>343.48±101.34</td>
<td>505.68±186.49</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>EIₘₓ</td>
<td>0.557±0.022</td>
<td>0.534±0.214</td>
<td>0.001</td>
</tr>
<tr>
<td>Critical time (median)</td>
<td>7.8</td>
<td>7.0</td>
<td>0.002</td>
</tr>
<tr>
<td>Acanthocytes (mean %)</td>
<td>4.48±2.06</td>
<td>6.61±2.18</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Echinocytes (mean %)</td>
<td>1.13±0.62</td>
<td>1.50±0.75</td>
<td>0.048</td>
</tr>
<tr>
<td>TBPI (median)</td>
<td>0.96</td>
<td>0.85</td>
<td>0.005</td>
</tr>
</tbody>
</table>
Chapter 5: Discussion

This study investigates the effect of oxidative stress and chronic inflammation on haemorheology. The study also looks at the association of altered haemorheology with abnormal peripheral arterial diseases (PAD) markers, thus linking altered haemorheology with CVDs. This chapter is divided into eight main sections, which discuss the major findings of the study. The effects of MetS, oxidative stress and chronic inflammation on haemorheology are discussed and the vascular effects of MetS in the form of elevated TBPI and D-dimer levels are presented in line with the altered haemorheology. The importance of altered haemorheology as a cardiovascular risk marker has been identified in this study and is presented in the final section of this chapter.

The outline of the chapter is:

5.1 Increased oxidative stress and chronic inflammation in metabolic syndrome
5.2 Dyslipidaemia and altered haemorheology
5.3 Metabolic syndrome and altered haemorheology
5.4 Oxidative stress, chronic inflammation and altered haemorheology
5.5.1 Erythrocyte morphology, oxidative stress, chronic inflammation and MetS
5.5.2 Techniques and parameters used in studying erythrocyte morphology
5.6. Association of oxidative stress and chronic inflammation with peripheral vascular diseases markers: ABPI and TBPI
5.7 Comparison of the association of altered haemorheology, peripheral vascular diseases markers and novel cardiovascular risk factors with metabolic syndrome
5.8 Clinical significance of the study: Altered haemorheology and cardiovascular diseases
5.1 Increased oxidative stress and chronic inflammation in MetS

Oxidative stress is defined as an imbalance between prooxidant and antioxidant factors in favour of the prooxidants (Sies, 1997). In order to look for evidence of oxidative damage in vivo, erythrocytes from patients with MetS and healthy controls were assessed for erythrocyte GSH, SOD and isoprostanes. This study reports increased oxidative stress in MetS groups when compared to healthy controls suggesting that the erythrocyte antioxidant defence mechanism is exhausted in MetS due to overproduction of reactive oxygen species. These observations are consistent with studies that have highlighted oxidative stress as a pathophysiological component of MetS (Furukawa et al., 2004; Sankhla et al., 2012; Yadav et al., 2015). The components of MetS (obesity, dyslipidaemia, hypertension and insulin resistance) have been individually associated with the oxidative stress in the previous studies (Bakker et al., 2000; Ceriello, 2000; Henriksen et al., 2011; Sankhla et al., 2012; Touyz, 2004). Increased oxidative stress has been suggested as a prior event in the development of MetS (Roberts & Sindhu, 2009) and it is likely to be a common second level abnormality in MetS (Grattagliano, Palmieri, Portincasa, Moschetta, & Palasciano, 2008).

Erythrocyte GSH and SOD level were decreased in the MetS group when compared to the control group (Table 4.5). The ubiquitous nature of the erythrocyte and the fact that it is an expendable cell (replaced on average every 120 days) makes it an ideal cell to protect other body tissues from free radical damage even at the expense of their own structure and function (Richards, Roberts, McGregor, et al., 1998). Erythrocytes play a significant role in scavenging free radicals (Mehlhorn, 1991; Richards, Roberts, Dunstan, McGregor, & Butt, 1998; Toth, Clifford, Berger, White, & Repine, 1984). In carrying out their role of free radical scavenging,
erythrocytes become damaged by oxidation, which consumes endogenous reducing substances, thereby reducing the level of erythrocyte antioxidant GSH and SOD. Reactive oxygen species superoxide anion crosses the erythrocyte membrane through a specific superoxide channel and is neutralised by the antioxidant SOD inside the erythrocyte (Lynch & Fridovich, 1978; Richards, Roberts, McGregor, et al., 1998). SOD is inactivated by its own product, hydrogen peroxide (Salo, Lin, Pacifici, & Davies, 1988). Hydrogen peroxide is broken down to water and oxygen by the enzyme catalase and glutathione peroxidase, the latter of which consumes GSH. Koziróg et al. (2011) similarly, observed that erythrocyte activity of SOD, GSH and catalase were lower in MetS participants compared with those in control participants.

Urinary isoprostanes level increased in the MetS group relative to that in the control group (Table 4.5), possibly due to the damage caused by free radicals in the MetS. Oxidative stress causes damage to critical biomolecules including DNA, lipids, and proteins (Floyd & Carney, 1992). Isoprostanes are formed by free radical-induced peroxidation of arachidonic acid (Morrow et al., 1990) and their measurement has been established as a reliable marker of oxidative stress status in vivo (Il'Yasova et al., 2015; Montuschi, Barnes, & Roberts, 2004). The observations from the present study were consistent with those of Hansel et al. (2004), who reported an increased concentration of plasma 8-isoprostanes level in MetS group when compared to that in control group. Elevated urinary isoprostanes level has been recently reported to be associated with several components of MetS (Costabile et al., 2015). Recently, Cangemi et al. (2012) have shown an increased concentration of platelets isoprostanes in the patients with type 2 DM and the authors have suggested that the over production of reactive oxygen species due to enhanced platelets NADPH oxidase isoform (NOX2) is responsible for increased
isoprostanes concentration in type 2 DM. Similarly, the urinary isoprostanes level was shown to be elevated in MetS group when compared to that in control group by Tsai et al. (2009) further supporting the findings from the present study. The elevated isoprostanes in MetS participants did not decrease even after a 4 to 5% weight reduction of the participants (Tsai et al., 2009). This is likely to be due to the fact that apart from weight (obesity) several factors, such as blood glucose level, dyslipidaemia and blood pressure interact in MetS and result in oxidative stress, and such factors are not necessarily normalised after weight loss. The median urinary isoprostanes level (corrected with urinary creatinine level) in healthy controls was 2.54 times higher than in MetS participants in the present study ($P<0.0005$). Hansel et al. (2004) found that, the plasma isoprostane level was 3.7 times higher in MetS participants compared to that in control group ($P<0.001$). Nevertheless, Hansel et al. (2004) used plasma whereas the present study used urine for the isoprostanes analysis. It has been suggested that the isoprostane value is overestimated in the plasma sample (Morrow, 2005), and therefore, this study used the urine sample for the estimation of isoprostanes level.

Contrary to the present study, no differences in the levels of urinary isoprostane were seen among control group, participants with one or two MetS risk factors and the MetS group in the study of Sjogren et al. (2005). The absence of any difference could have been due to the small number of MetS participants ($n=22$) in their study (Sjogren et al., 2005). More importantly, the different severity of DM, hypertension and dyslipidaemia in MetS participants in different groups could have played a role in the different observations. Among the 36 MetS participants in the present study, 22 had DM, 29 had hypertension, 23 had hypertriglyceridaemia, 19 had low
HDL-C and 12 had high waist circumference. In comparison, the severity of the dyslipidaemia in the study of Sjogren et al. (2005) was lower when compared to that in the present study.

The present study has also demonstrated an increased concentration of hsCRP in the MetS group relative to that in the control group (Table 4.5), suggesting the presence of low-grade systemic inflammation among the participants with MetS. This chronic low-grade inflammation has been hypothesised to play a critical role in the development of MetS (Dandona et al., 2005). Low grade systemic inflammation as evidenced by high levels of hsCRP in blood is suggested to be one of the mechanisms by which known risk factors such as obesity, smoking and hypertension promote the development of type 2 DM (Pradhan, Manson, Rifai, Buring, & Ridker, 2001). Elevated TNF-α and Interleukin-6 (IL-6) in MetS participants stimulate the production of hsCRP by the liver (Pickup, Mattock, Chusney, & Burt, 1997), increasing its concentration in circulation. The present finding is in agreement with the studies that have confirmed the association of hsCRP with MetS (Kang et al., 2005; Ridker et al., 2003; Shrestha et al., 2011; Sigdel et al., 2014).

In this study, the median level of hsCRP was higher in group III (MetS group) than in group II (one or two components positive) participants (refers to 4.2.3). A previous study has reported a linear increase in the median CRP levels along with increasing MetS (Ridker et al., 2003) in agreement with the present findings. Furthermore, hsCRP was shown to provide the additional prognostic information in terms of future cardiovascular risk, at all levels of severity of MetS (Ridker et al., 2003). The American Heart Association suggested that hsCRP measurements might provide information for a risk assessment for coronary heart disease beyond that obtained
from the established risk factors (Pearson et al., 2003). It has also been suggested that hsCRP could be used as a marker for the primary prevention of CVDs (Ridker et al., 2003), and it has been shown that the risk of cardiovascular events doubles in MetS with hsCRP > 3.0 mg/L compared with in MetS participant with hsCRP < 3.0 mg/L (Ridker et al., 2003). The findings and observations in this study suggest that chronic inflammation, as indicated by elevated hsCRP level, plays a critically important role in the pathophysiology of MetS and is also a major determinant of future CVDs.

The present study provides evidence that oxidative stress and chronic inflammation are present in MetS. These two parameters are linked to the progression of CVDs (Ceriello & Motz, 2004; Ridker et al., 2003). The extent to which these two parameters affect blood cells and haemorheology, and the way that the altered haemorheology is associated with vascular diseases is discussed in the following sections.
5.2 Dyslipidaemia and altered haemorheology

Plasma lipid concentrations may affect lipid composition of erythrocyte membrane (Dougherty, Galli, Ferro-Luzzi, & Iacono, 1987), which may subsequently alter erythrocyte morphology and other rheological characteristics. This study examined the possible effect of dyslipidaemia on haemorheology. Research participants were divided into normolipidaemic and dyslipidaemic groups and the differences in the values of the haemorheological parameters between these two groups were analysed. This study demonstrated significant differences in erythrocyte aggregation between normolipidaemic and dyslipidaemic groups (Table 4.6). Erythrocyte aggregation was associated with abnormalities in lipid parameters, and the associations of HDL-C and triglyceride were stronger than those for LDL-C and total cholesterol. Similarly, erythrocyte aggregation was negatively correlated with HDL-C and positively correlated with Triglyceride and LDL-C (Table 4.7).

The findings support the proposal that there are some underlying processes related to dyslipidaemia that are relevant to erythrocyte aggregation. LDL-C enhances erythrocyte interaction and increases erythrocyte aggregation (Crowley et al., 1994; Simon et al., 1995). The interaction of LDL-C with erythrocyte membrane is competitively reduced by HDL-C, thus decreasing erythrocyte aggregation (Sloop & Garber, 1997). This plausible mechanism reinforces the negative effect of LDL-C and positive effect of HDL-C on the cardiovascular system. It has been hypothesised that changes in the lipid composition of plasma and the erythrocyte membrane may enhance the interaction between erythrocytes and plasma proteins, and thus, potentially results in hyperaggregation (Cicha et al., 2001; Maeda et al., 2006). It has been shown that plasma lipid composition determines the aggregation properties of erythrocytes.
(Terano et al., 1983), and several studies have associated dyslipidaemia with erythrocyte aggregation in agreement with the findings of this study (Abrashkina et al., 2010; Babu, 2009a). Increased erythrocyte aggregation could form thrombi at low shear rate that may lead to ischemia and infarction (Stoltz & Donner, 1987). Steinberg et al. (2000) demonstrated that after eight hours of infusion of intralipid and heparin in insulin-sensitive lean participants, there was a reduction in blood flow by about 50%. Reduction in blood flow was attributed to the impairment of the release of endothelium-derived nitric oxide due to elevated free fatty acids concentration, which in turn reduces insulin mediated vasodilation (Steinberg et al., 2000). There is also a possibility that elevated level of circulating free fatty acids increases erythrocyte aggregation which suppresses expression of nitric oxide synthesising mechanisms (Baskurt, Yalcin, Ozdem, Armstrong, & Meiselman, 2004) further reducing insulin mediated vasodilation.

The present study found that the mean percentage of acanthocytes was higher in dyslipidaemic groups than that in normolipidaemic group. The high plasma concentration of the lipids seen in dyslipidaemia results in deposition of the lipids in the erythrocyte membrane (Schick & Schick, 1985), thereby altering erythrocyte shape. Positive correlation of acanthocytes with triglyceride and negative correlation with HDL-C in this study confirms that plasma lipid concentrations have a negative effect on the lipid composition of the erythrocytes as suggested by Dougherty et al. (1987) and the extent of the alterations of erythrocyte lipid composition could possibly determine its morphology. Furthermore, when the concentration of lipids is high in the plasma, their deposition on the erythrocyte membrane increases stiffness of the membrane (Kanakaraj & Singh, 1989). The present study similarly found that maximum erythrocyte deformability was negatively correlated with Triglyceride and positively correlated with HDL-C. The inverse
relationship of HDL-C with unfavorable haemorheology could be due its antioxidative (Tribble, Chu, Gong, van Venrooij, & Nichols, 1995) and anti-inflammatory properties (Barter et al., 2004) of HDL-C. The results of this study are supported by studies that have shown the effect of increased concentration of harmful lipids and FFA in the normal morphology of erythrocytes (Babu, 2009b; Jozwiak, Palecz, & Bartosz, 1994; Zavodnik et al., 1997). Increased low shear rate WBV is typically secondary to increased erythrocyte aggregation (Chien et al., 1967). However, this study did not observe differences in WBV between normolipidaemia and dyslipidaemia groups, and no significant association of dyslipidaemia was seen with hyperviscosity. This is possibly explained by the measurement of WBV at the high shear rate (150 s⁻¹). Carallo et al. (2013) also reported that no difference in WBV between participants with low and normal HDL-C levels where WBV was measured at high shear rate (225 s⁻¹) supporting the findings of the present study.

ROC curve (Table 4.9) and regression (refer to 4.3.4) analyses showed that, among the lipid parameters; triglyceride and HDL-C were significantly associated with erythrocyte aggregation. Although the association of aggregation parameters and dyslipidaemia was not very strong, the significant association suggests the importance of triglyceride and HDL-C in haemodynamics. Several studies have demonstrated increased level of erythrocyte aggregation due to elevated triglyceride (Cicha et al., 2001; Zhao et al., 2006). Vayá et al. (2004) showed that, among plasma lipids, triglyceride predominantly was associated with increased erythrocyte aggregation in survivors of acute myocardial infarction, supporting the findings of the present study. There is controversy about whether triglyceride is directly related to atherosclerosis and/or CVD (Hulley, Rosenman, Bawol, & Brand, 1980). The significant association of triglyceride among lipid
parameters with erythrocyte aggregation in the present study suggests that the possible effect of triglyceride in the cardiovascular system cannot be ignored. Patients who have achieved a LDL-C target goal could still remain at high risk for cardiovascular events if they have elevated triglyceride and low HDL-C (Barter et al., 2007; Miller et al., 2008). The American Heart Association has considered triglyceride to be an independent cardiovascular risk factor (Miller et al., 2011), and similarly, HDL-C has been considered to be an independent CVD risk factor (Boden, 2000). Hence, the European Atherosclerosis Society Consensus Panel recommended elevated plasma triglyceride, and similarly, low HDL-C levels as a target for treatment (Chapman et al., 2011). The association of HDL-C with myocardial infarction was found to be stronger than that of LDL-C in a study by Sigdel et al. (2012), possibly indicating that HDL-C is the major determinant of clinical outcomes. The findings from the present study emphasise that HDL-C and triglyceride should be given at least equal, or if not more, importance than other routinely measured lipid parameters in the context of blood rheology and CVDs.
5.3 Metabolic syndrome and altered haemorheology

Erythrocyte deformability (EImax and SS1/2), WBV and erythrocyte aggregation (critical time and critical stress) were measured in this study to investigate the possible effect of MetS on haemorheology. The results show that haematocrit adjusted WBV and erythrocyte aggregation were higher, whereas erythrocyte deformability (EImax) was lower, in participants with MetS than in participants without MetS (Tables 4.10, 4.11 and 4.12). The findings indicate that MetS participants have an abnormal blood rheological profile that may complicate the progression and manifestation of MetS. These findings are also in agreement with observations of Vaya et al. (2011), who reported higher corrected WBV, increased erythrocyte aggregation and reduced erythrocyte deformability in patients with MetS than in participants without MetS but who had one or two components of MetS. Likewise, Presti et al. (2002) reported decreased erythrocyte deformability and increased WBV in participants with MetS compared to healthy controls.

Furthermore, this study showed that WBV was significantly higher in MetS participants (Group III) than in participants with only one or two positive MetS components (Group II) (refer to 4.4.2). Mean concentration of WBV has been reported to be significantly increased \((P<0.01)\) with the increasing components of MetS (Zhang, Pu, Zhang, & Ren, 2006), supporting the finding of the present study. Similarl, Irace, Scavelli, Carallo, and Serra (2009) reported higher haematocrit adjusted WBV in participants with MetS compared to those without MetS, and WBV increased with the increasing number of the MetS components.

Erythrocyte aggregation was found to be higher in the groups with one or more positive MetS components (Group II and Group III) than in the control group (Group I), and highest in the
group with three or more positive components (Group III) (refer to 4.4.4). Toker et al. (2005) similarly reported a significant increase in mean erythrocyte aggregation percentage ($P=0.001$) with an increasing number of MetS components. The findings of the present study along with past observations suggest that an alteration in haemorheology is correlated with the severity of the MetS (increased number of components). They also suggest that alterations in haemorheology have a direct effect on the pathophysiology of MetS. Obesity has been considered as the central component that affects blood rheology and blood pressure (Bogar, 2002). Central obesity and a lack of physical exercise are the main factors responsible for insulin resistance (Kahn & Flier, 2000), and the combined effects associated with insulin resistance and obesity may lead to the alterations in haemorheology and the development of MetS (Brun, Aloulou, & Varlet-Marie, 2004).

In general, the haemorheological parameters were significantly correlated with the components of MetS in the present study (Table 4.15). Critical time and critical stress were correlated with all five of the components of MetS, suggesting that erythrocyte aggregation is correlated with MetS. With the exception of systolic blood pressure, $E_{\text{max}}$ was correlated with all of the components of MetS. $SS_{1/2}$ was negatively correlated only with HDL-C. Likewise, WBV was positively correlated with systolic BP, waist circumference and FBG. The cardiovascular risk factors, age and haemoglobin-adjusted correlation were noted between the degree of erythrocyte aggregation and the number of the components of MetS in the study by Toker et al. (2005), supporting the present findings. Furthermore, other studies have shown that blood viscosities at different shear rates were correlated with waist:hip ratio (obesity) and high blood pressure (Presti et al., 2002). Highest quartile WBV measurement was positively associated with waist circumference and
negatively with HDL-C (Zhang et al., 2006). Nevertheless, correlation does not necessarily indicate the relationships among variables nor does it indicate the direction of interaction. Therefore, this study undertook regression analysis among MetS and rheological variables to determine the relationships among variables (predictor and response variables).

Haemorheological parameters were divided into four quartiles by using 25\textsuperscript{th}, 50\textsuperscript{th} and 75\textsuperscript{th} percentiles (Table 4.8). MetS was significantly associated with the highest quartile of critical stress and WBV, and with the lowest quartile of erythrocyte deformability (refer to 4.4.11). The association of MetS with the unfavourable rheological quartile supports the previous results, which suggested that altered haemorheology may have some role in determining the complications arising from MetS. In the present study, ordinal logistic regression analysis showed that the highest quartile of WBV values occurred 7.958 (95\% CI, 2.556 to 24.775) times more frequently than the lowest quartile values in patients with MetS ($\chi^2(1)=12.816$, $P<0.0005$). However, Zhang et al. (2006) reported that the highest quartile of WBV (hyperviscosity) occurred only 4.8 times more frequently than the lowest quartile measurement among patients with four MetS components. The relatively high frequency in the occurrence of hyperviscosity in MetS patients in the present study compared to that observed by Zhang et al. (2006) could be explained by differences in the groups of MetS patients recruited. In this study, the mean age of the MetS patients was 63.06±12.13 years, and six out of thirty-six MetS patients had all five components positive. By contrast, all of the MetS patients in the study by Zhang et al. (2006) were active office workers below the age of 59, and none of them had all five components.
Regression analysis showed that none of the MetS components predicted hyperviscosity or low deformability, but that all of the individual components of MetS predicted erythrocyte hyperaggregation (Tables 4.16, 4.17 and 4.18). Similarly, Vaya et al. (2011) reported that none of the MetS constituents independently predicted native WBV, but that erythrocyte hyperaggregation was significantly predicted by all of the MetS constituents except high blood pressure. The findings from the correlation and regression analyses with erythrocyte aggregation in this study indicate that individual MetS components may have an effect on rheological parameters. Several studies have demonstrated the effect of individual MetS components on rheological parameters in agreement with the findings of this study (Husstedt et al., 1997; Medvedev & Skoriatina, 2012; Pirrelli, 1999; Solá et al., 2007).

More importantly, the present study indicated that the individual MetS components did not predict increased WBV and low erythrocyte deformability, but that these rheological components predicted MetS even after they were adjusted for the effects of age, sex and DM. Such observations indicate that the individual MetS components may not directly affect WBV and erythrocyte deformability, even though their combined effect in the form of MetS is significant. This result suggests that the complex interplay of MetS components is clinically more important than the effects of individual components. It supports the whole concept of MetS i.e. individuals with three or more positive components are at increased risk of heart diseases compared with those individuals with only one or two positive components. Even after adjusting for the effect of DM, MetS was predicted by all of the rheological components, indicating that insulin resistance is not the only cause for haemorheology alterations in MetS. The concurrences of the components of MetS probably have a cumulative effect in enhancing alterations in
haemorheological parameters. The complexity of the interrelationships between the various components of MetS and its haemorheological manifestations is clearly emphasised by the present findings.
5.4 Oxidative stress, chronic inflammation and altered haemorheology

This study reports increased levels of oxidative stress and chronic inflammation, and unfavourable haemorheology in MetS participants compared with healthy controls. One of the main objectives of the study was to investigate the hypothesis that the increased oxidative stress and chronic inflammation present in MetS negatively affect the rheological components of blood, and the findings show that the altered haemorheology is associated with the chronic inflammation and oxidative stress present in MetS.

Erythrocyte GSH and SOD were negatively correlated with erythrocyte aggregation and WBV, whereas erythrocyte SOD was negatively correlated with erythrocyte aggregation (Table 4.19). These results appear to support the proposition that the increased erythrocyte aggregation is due to the oxidative damage of erythrocytes. The labile groups in the proteins and lipids of the erythrocyte cytoskeleton are oxidised due to the decreased antioxidant level, and this oxidative modification of membrane proteins and lipids possibly increases the tendency of ‘damaged’ erythrocytes to adhere to other erythrocytes, thereby increasing erythrocyte aggregation. Richards and Nwose (2010) also observed increased erythrocyte oxidative stress with increased WBV in different stages of diabetes pathogenesis. Abnormal WBV was linked to oxidative stress in 76% of the apparently healthy controls (Richards & Nwose, 2010). Intravenous infusion of reduced glutathione significantly reduced blood viscosity and erythrocyte aggregation in atherosclerotic patients (Coppola et al., 1991), supporting the suggestion from the present study that the depletion of antioxidant is associated with alterations in haemorheological profile.
Erythrocyte deformability was negatively correlated with urinary isoprostanes, but it was not correlated with either erythrocyte GSH or SOD (Table 4.19). Erythrocyte deformability appears to be affected by the accumulation of oxidative stress end-products and lipid peroxidation rather than the free radical, but this aspect needs further clarification. Erythrocyte deformability has been also shown to be negatively correlated with malondialdehyde, another end-product of lipid peroxidation (Bekyarova, Yankova, Kozarev, & Yankov, 1996). Malondialdehyde can cause polymerisation of membrane components, and thus, increase the aminophospholipid bilayer rigidity (Chiu, Kuypers, & Lubin, 1989; Jain, Mohandas, Clark, & Shohet, 1983). Furthermore, the peroxidant injury initiated in the lipid component of the membrane can be transmitted to neighboring molecules such as membrane proteins. These polymerisation reactions subsequently increase membrane rigidity. The increase in WBV associated with elevated malondialdehyde has been attributed to the protein cross-linking and binding capacity of malondialdehyde (Liu, Qin, & Yin, 2004). In the present study, urinary isoprostanes, the markers of oxidative lipid damage, significantly predicted alterations in haemorheology (Table 4.20), and were positively correlated with WBV and erythrocyte aggregation, and negatively correlated with erythrocyte deformability. However, the prediction of altered haemorheological profile (WBV and critical stress) by oxidative stress and inflammatory markers, though significant, was weak (refer to figure 4.2). The significant trend ($P<0.05$) with small $R^2$ value indicates that the predictor variable (oxidative stress and inflammation) still provides some information about the response (haemorheology in our case) even though data points fall further from the regression line. This suggests that though the oxidative stress and chronic inflammation could predict the changes in the rheological parameters, the predictions are not precise and there are lot of other co-variables that also affect the rheological parameters. Many metabolic abnormalities (hyperglycaemia,
dyslipidaemia, blood pressure, abnormal plasma proteins concentration) along with oxidative stress and inflammation could affect the haemorheology. Though our data supports the hypothesis that generation of oxidative stress and inflammation is unfavourable for blood rheology, to find out the precise pathway and degree of effect, larger studies should be conducted with clinically homogenous population. Kowalczyk et al. (2012) also reported an unfavorable alteration in erythrocyte lipid membrane fluidity in MetS participants. Malondialdehyde concentration in erythrocytes in MetS patients was higher than that in healthy controls, but no significant changes in the activity of glutathione peroxidase, catalase and SOD were seen. Membrane lipid peroxidation was considered a possible cause of the altered membrane properties (Kowalczyk et al., 2012), in agreement with the findings of the present study.

In this study, some of the research participants in the MetS group took anti-inflammatory drugs. Nevertheless, hsCRP level was higher in the MetS group compared with that in the control group. Erythrocyte aggregation was significantly associated with the inflammatory marker, hsCRP. The increased aggregation of erythrocytes in the MetS group observed could be due to the elevated inflammation as evidenced by the increased hsCRP level. This suggests that increased levels of adipocytokines, inflammatory molecules and acute phase proteins generated in MetS negatively affect the normal erythrocytes by increasing their tendency to aggregate. Increased erythrocyte aggregation could be due to the presence of increased levels of adhesive proteins (such as adipocytokines and acute phase proteins) in their milieu. Erythrocyte aggregation was correlated with all five MetS components in this study. This is also likely to be due to the excessive generation of inflammatory molecules in MetS (Al Rifai et al., 2015; Toker et al., 2005). The aggregability of the cells might reflect the low grade chronic inflammation of
MetS (Schechner et al., 2003), and in fact, erythrocyte aggregation has been shown to be a better indicator of inflammation than ESR and fibrinogen level in MetS (Toker et al., 2005).

Overall, this study suggests that the important factors that influence rheological parameters are oxidative stress and inflammation. Several studies have associated oxidative stress (Cicha, Suzuki, et al., 1999; Hierso et al., 2014; Marotta et al., 2001; Zhang et al., 2010) and chronic inflammation (Assayag et al., 2005; Samocha-Bonet et al., 2003) with abnormal haemorheological state in agreement with the findings of the present study. Elishkevitz et al. (2002) reported that erythrocyte aggregation was associated with inflammatory markers, but not with glycosylated haemoglobin. Similarly, the decrease in erythrocyte aggregation in patients with type 2 DM after intense management was not shown to be correlated with the changes in glycaemic control, fibrinogen or lipid profile (Chong-Martinez, Buchanan, Wenby, & Meiselman, 2003). Foresto et al. (2002) reported that erythrocyte rigidity and aggregation increased in patients with essential hypertension, irrespective of differences in fibrinogen concentration and WBV. Simmonds et al. (2012) on the other hand, demonstrated an improvement in haemorheology in diabetic patients after twelve weeks of exercise without any concomitant improvement in glycaemic control. These studies support the findings of the present study in suggesting that alterations in erythrocyte intrinsic properties due to the oxidative stress and chronic inflammation may be the main factors (rather than dyslipidaemia, glycaemia and high fibrinogen level) for increased erythrocyte aggregation.
5.5.1 Erythrocyte morphology, oxidative stress, chronic inflammation and MetS

The present study investigated the morphological changes in erythrocytes in MetS and tested the hypothesis that the morphological changes are associated with oxidative stress and chronic inflammation. The results indicated that MetS participants had a greater prevalence of erythrocytes with abnormal morphology (acanthocytes, echinocytes and stomatocytes) and a lower prevalence of biconcave shape erythrocytes compared with healthy controls (Table 4.21). This is likely to be due to the continuous attack of reactive oxygen species, which consume endogenous reducing substances of erythrocytes. Oxidative attack, through oxidation of labile groups in the proteins of the cytoskeleton, damage erythrocytes by altering their lipid bilayer (peroxidation), subsequently resulting in shape change. It has been suggested that treatment of physiological biconcave discoid erythrocytes with various agents can transform them into their two extreme opposite forms: stomatocytes and echinocytes (Deuticke, 1968). The findings of this study are in agreement with those from Straface et al. (2011), which reported an increase in the number of acanthocytes in MetS participants compared with healthy controls. Another similar study, reported diabetic patients to have higher number of ‘acanthocytes’ and ‘stomatocytes’ in comparison to non-diabetic participants, which after treatment with antioxidant drug was restored to normal (Straface et al., 2002). The restoration of morphologically abnormal cells to normal after antioxidant treatment highlights the effect of oxidant on blood cells.

In the present study, isoprostanes and hsCRP were negatively correlated with biconcave cells and positively correlated with acanthocytes (Table 4.23). On the contrary, GSH was positively correlated with biconcave cells and negatively correlated with acanthocytes (Table 4.23). This suggests that an important link may exist between erythrocyte morphology, and oxidative stress
and chronic inflammation. Erythrocytes may respond by changing their morphology to any form of insult in their membrane or biochemical composition. The findings in this study are consistent with past observations (Snyder et al., 1985) that have demonstrated the conversion of normal erythrocyte populations to echinocytes after incubation with hydrogen peroxide. The mechanism of echinocyte formation may be related to condensation of the inner monolayer lipids as a result of spectrin-haemoglobin complex formation due to oxidative damages (Snyder et al., 1985).

Crenated forms of erythrocytes were observed in a dose-dependent manner when blood samples from healthy donors were incubated with peroxynitrite (Starodubtseva, Tattersall, et al., 2008). The authors suggested that crenation of erythrocytes appears to be caused by both water and ion imbalance between the cells and surrounding medium, and that cytoskeletal structure changes as a result of oxidative stress (Starodubtseva, Tattersall, et al., 2008). These studies are in agreement with the present study in suggesting that erythrocyte morphology is affected by oxidative stress. The increased count of abnormal erythrocytes observed in the MetS group in the present study could be due to the generation of oxidative stress in MetS.

MetS components, such as hypertriglyceridaemia, hypertension and central obesity, were positively correlated with one or more of the other morphologically altered cells (acanthocytes, echinocytes and stomatocytes) and negatively correlated with biconcave cells. Plasma HDL level, however, as expected, was positively correlated with biconcave cells and negatively correlated with acanthocytes. In the concept of MetS, dyslipidaemia refers to high triglycerides and/or a low HDL-cholesterol level (Expert Panel on Detection Evaluation Treatment of High Blood Cholesterol in Adults, 2001). High plasma triglyceride levels could be cytotoxic to the erythrocytes and potentially change their shape. Other studies have shown that increased
concentrations of harmful cholesterol and FFA alter the normal morphology of the erythrocytes, in agreement with this study (Babu, 2009b; Jozwiak et al., 1994; Zavodnik et al., 1997). When the concentration of cholesterol is high in plasma, it is deposited on the erythrocyte membrane (Schick & Schick, 1985). This increases the stiffness of the membrane and alters the erythrocyte shape (Kanakaraj & Singh, 1989), thus reducing its deformability. A study that determined erythrocyte membrane parameters in components of MetS, found that all membrane parameters differed significantly in MetS participants compared with control groups. It was concluded that erythrocyte membrane structural changes are associated with features of MetS (Anichkov, Maksina, & Shostak, 2005), which is consistent with observations from the present study.

The study of erythrocyte morphology is of great importance in haemorheology, since the deformability of circulating cells has a fundamental influence on rheological properties of the blood (Dintenfass, 1971). The present study showed a positive correlation between biconcave cells and erythrocyte deformability (Table 4.22). The principal role of erythrocytes is to transport gases, namely oxygen, carbon dioxide and nitrous oxide. Erythrocytes are highly specialised for this task, losing most of their organelles in development, and containing mostly haemoglobin. Their biconcave shape permits flexibility, allowing them to squeeze through capillaries narrower than their own diameter. This is supported by the positive correlation between biconcave cells and erythrocyte deformability observed in this study. More importantly, the significant negative correlation between percentage of acanthocytes and erythrocyte deformability in the present study indicates that changes in cell morphology due to oxidative stress and chronic inflammation lead to the generation of rigid cells, possibly compromising the rate of blood flow through capillaries and oxygen delivery to peripheral organs. Watanabe et al. (1990) showed that
erythrocytes from healthy donors incubated in an hypoxanthine/xanthine oxidase system and hydrogen peroxide had decreased membrane fluidity, and hence, reduced deformability in agreement with the findings of the present study.

5.5.2 Techniques and parameters used in studying erythrocyte morphology

Variations in the morphology of erythrocytes are observed in various pathologies such as anaemia and thalassaemia, but such variations have not been used as markers in the diagnosis and management of metabolic diseases like CVD, DM or MetS. Although several studies have addressed the importance of altered erythrocyte morphology in metabolic diseases, the use of different techniques has resulted in conflicting evidence. The present study investigated erythrocyte morphology using SEM, since several studies in the past have used the same technique (Snyder et al., 1985; Watanabe et al., 1990). In comparison, erythrocyte morphology was studied using a light microscope with a 100X immersion lens by Turchetti et al. (Turchetti et al., 1999; Turchetti et al., 1998; Turchetti et al., 1997). Similarly, Cimbaljevic et al. (2007) used light microscopy after May Grunwald-Giemsa staining to study erythrocyte morphology. Light microscopy requires the air-dried and the methanol fixation at a microscopic slide, which have the potential to introduce artifact. Also, it is not sensitive to minimal morphological changes.

The nomenclature given by Bessis (1972) is accepted as the gold standard in erythrocyte morphology studies, and therefore, the present study followed the same classification. An example of the cells that this study considered is shown in Figure 5.1. In the present study, normal disc shaped cells are referred to as ‘biconcave cell’. When erythrocytes are washed in isotonic saline and viewed on a glass slide under a coverslip, biconcave cells are transformed to
sphere cells (‘echinocytes’) covered with crenations or spicules. ‘Acanthocytes’, though superficially resembling echinocytes, are considered to be different cells with fewer spicules (which are irregularly arranged and are bent back at their tips) on their surface. The cup shaped cells are referred to as ‘stomatocytes’ and spherical cells are referred to as ‘spherocytes’. However, not all studies have used this system, and the use of different nomenclature systems for the identification of changes in erythrocyte morphology has resulted in the conflicting results of erythrocyte morphology in metabolic diseases. For instance, Turchetti et al. (1999; 1998; 1997) used the term ‘bowl shaped’ for most deformable cells and discocytes (biconcave), though referred as physiological cells, were considered less deformable than bowls. Other studies (Starodubtseva, Kuznetsova, Yegorenkov, & Cherenkevich, 2008; Straface et al., 2002) considered biconcave shape as the gold standard for morphologic comparisons without taking ‘bowl shape’ into account. ‘Spiculed cells’ with surface projections, or echinocytes, are interchangeably referred to as crenated cells or acanthocytes by different authors (Williams, Beutler, Erslev, & Rundles, 1972), whereas the present study clearly differentiates between acanthocytes and echinocytes. Watanabe et al. (1990) used the term ‘spiculated form’, Starodubtseva et al. (2008) used the term ‘crenated form’, and Straface et al. (2002) used the term ‘acanthocytes’, and all these cells probably referred to echinocytes. ‘Area’, ‘perimeter’ and ‘form factor’ has also been considered to discuss erythrocytes morphologic changes (Babu, 2009b; Babu & Singh, 2004).

Different sample preparation techniques and components also affect the erythrocyte morphology. The present study used glutaraldehyde as a cell fixative immediately after collection (refer to 3.4.5) so that the morphology of cells was preserved and not changed due to storage. This
method of fixation has the least potential to artefactually change the erythrocyte shape. The low concentration of glutaraldehyde helps to restore the normal biconcave-shape of erythrocytes (Eriksson, 1990). During sample preparation, erythrocytes can be transformed into echinocytes when located between glass surfaces. This ‘glass effect’ depends on the composition of the glass surface, the distance between the two adjacent surfaces, haematocrit and temperature (Eriksson, 1990). The glass effect is minimal with the glutaraldehyde fixative and SEM. Nevertheless, it should still be noted that there is a slight chance that the increased concentration of acanthocytes obtained in the present study may be at least partially due to this glass effect.

**Figure 5.1** Erythrocyte morphological variation identified from a scanning electron microscope: (a) biconcave cell (1500x); (b) acanthocyte (1500x); (c) echinocyte (1500x); (d) echinocyte (5000x) and (e) stomatocyte (5000x).
5.6 Association of oxidative stress and chronic inflammation with peripheral vascular disease markers: ABPI and TBPI

ABPI and TBPI are non-invasive cardiovascular risk markers used to assess peripheral vascular diseases, and hence, are indicators of PAD. This study demonstrated an increase in the value of ABPI in the MetS group relative to that in the healthy control group (Table 4.24). The increase in the value of ABPI in the MetS group could have been due to long term diabetes complications in MetS participants in the present study.

Ankle blood pressure readings often go beyond 200 mm Hg due to the calcification of lower limb arteries in DM, giving a falsely high ankle pressure (Brooks et al., 2001). Therefore, ABPI is not considered as a useful marker for vascular assessment if calcification of arteries has already progressed (Strandness & Bell, 1965) as in the case of DM. For this reason, this study also measured TBPI to assess the vascular functions correctly in the MetS group and compared them with those in the healthy control group. The results showed decreased TBPI in the MetS group relative to that in the healthy control group (Table 4.24). TBPI is commonly used for assessment of vascular functions (Mayfield et al., 1998; Vowden, Goulding, & Vowden, 1996). A decreased value of this parameter in the MetS group in the present study indicates abnormal blood flow in larger and smaller arteries. Such observations indicate that MetS participants are at risk of CVDs, because TBPI has been shown to predict cardiovascular morbidity (Ono et al., 2003). A high prevalence of peripheral vascular diseases has been noted in patients with MetS (Gorter et al., 2004; Olijhoek et al., 2004). TBPI and ABPI were significantly associated with DM in this study in agreement with the findings of other studies (Spångéus et al., 2013; Tapp et al., 2003).
Given that oxidative stress and chronic inflammation are novel cardiovascular markers, this study investigated the possible association between non-invasive PAD markers and these cardiovascular markers. The results showed that abnormal TBPI was predicted by SOD, GSH and isoprostane levels, while abnormal ABPI was predicted only by GSH level (Table 4.29). Similarly, TBPI showed a linear increase across the quartiles of erythrocyte SOD and GSH and a linear decrease across the quartiles D-dimer, and in the case of ABPI, this trend (linear decrease) was only followed across the quartiles of GSH. These results support the idea of there being some underlying processes related to oxidative stress, which are relevant to PAD. The decrease in blood flow may be associated with the generation of oxidative stress in the body. The findings of the present study underscore the potential mechanism of the oxidative stress–driven progression of peripheral atherosclerotic disease. However, though the prediction was significant ($P$-value <0.05), low $R^2$ values suggest that the size of prediction is weak. This indicates that there are other variables that primarily determine the initiation and progression of the atherosclerotic lesions. The predictive value of the ABPI in cardiovascular morbidity and mortality was found to be similar to that of traditional Framingham risk factors (Bhasin & Scott, 2007). Recently, ABPI was shown to correlate with global atheroma burden based on measurements by whole body contrast enhanced magnetic resonance angiography in 50 patients with symptomatic PAD (Weir-McCall et al., 2014). Several studies have suggested that ABPI is an indicator of CVD (Ankle Brachial Index Collaboration et al., 2008; Newman et al., 1999; Newman et al., 1993; Olijhoek et al., 2004; Vogt et al., 1993).
There was no linear increase or decrease in ABPI and TBPI across the quartiles of inflammatory marker hsCRP (Table 4.31). The inflammatory marker, hsCRP, was not correlated with ABPI and TBPI, and did not significantly predict decreased ABPI or TBPI (Tables 4.28 and 4.29). Such findings indicate that PAD does not have a clear association with systemic chronic inflammation. Musicant et al. (2006) observed that CRP was not associated with the progression of PAD in agreement with the finding of the present study. Folsom et al. (2001) reported that CRP was not associated with carotid intima-media thickness after adjusting for established vascular risk factors, and they argued that CRP may be the marker of thrombotic risk rather than the degree of atherosclerosis. Similarly, Hunt et al. did not find an association between hsCRP and calcified subclinical atherosclerosis, and suggested that inflammation may represent the atherosclerotic disease process rather than the disease burden (Hunt, O'Malley, Vernalis, Feuerstein, & Taylor, 2001). It has been pointed out that inflammation and oxidative stress function differently in the pathogenesis of atherosclerosis, with the former leading mainly to endothelial dysfunction and the later responsible for arterial stiffening (Kals et al., 2008). The Centre for Disease Control and Prevention and American Heart Association scientific statement also suggests that inflammatory markers may measure ongoing ulceration and thrombosis rather than the atherosclerotic mass (Pearson et al., 2003).

One observation worthy of emphasis from the present study is that ABPI was associated only with GSH level, but not with SOD, isoprostanes, hsCRP or D-dimer levels. The poor association of ABPI with emerging markers of cardiovascular risk factors (oxidative stress and chronic inflammation) suggests that it cannot be used (or at least, on its own) as a CVD risk marker. Similarly, Rosoky et al. (2010) found that ABPI was not associated with oxidized-LDL-C.
Oxidized LDL is an oxidative stress marker that is formed by the oxidation of LDL and subsequently phagocytosised by macrophages during the progression of atherosclerosis (Esterbauer, Gebicki, Puhl, & Jurgens, 1992). To the best of my knowledge, no study has reported the association of TBPI with oxidative stress markers. The present study demonstrates the association of non-invasive PAD markers, ABPI and TBPI, with oxidative stress, but the association of ABPI was found to be poorer than that of TBPI.

In contrast to the present findings, several studies have shown a significant association of hsCRP either with ABPI or TBPI (Abdellaoui & Al-Khaffaf, 2007; Aso et al., 2004; Tajiri, Mimura, & Umeda, 2005). These differences could be due to different numbers of participants and more importantly, due to a clinically different cohort (diseases, duration, on-going thrombosis or already established vascular lesion) of study participants. Inflammation and oxidative stress play a key role in the pathophysiology of atherosclerosis (Harrison, Griendling, Landmesser, Hornig, & Drexler, 2003; Pearson et al., 2003), but the ‘cause or effect’ relationship between these two needs further research. It has been suggested that chronic inflammation is possibly a cause while oxidative stress is a consequence of atherosclerosis (Stocker & Keaney Jr, 2004). We believe that inflammation generated from any cause of cellular damage (e.g. infection, autoimmunity, and trauma) causes oxidative stress which leads to further cellular damage and inflammation in a self-perpetuating sequence. Oxidative stress is also believed to be the mechanism that initiates inflammation in obesity and MetS (Brownlee, 2001), and contributes to endothelial dysfunction and CVD (Heitzer, Schlinzig, Krohn, Meinertz, & Münzel, 2001). A large-scale study on inflammatory and oxidative stress markers needs to be undertaken to better understand the relationship among chronic inflammation, oxidative stress and vascular lesions in PAD.
5.7 Comparison of the association of altered haemorheology, peripheral vascular diseases markers and novel cardiovascular risk factors with MetS

This study measured haemorheological parameters, PAD markers, oxidative stress and chronic inflammation in MetS participants, and compared the results for these attributes with those in both healthy control group and in participants having only one or two positive MetS components.

The age- and sex-adjusted odds ratio for predicting MetS was higher for haemorheological parameters when compared to TBPI (Table 4.32). After adjusting for the effects of DM, age and sex, ABPI and TBPI were not associated with MetS (Table 4.33). However, this was not the case with haemorheological parameters. Even after adjusting for the effects of age, sex and DM, the odds ratios for predicting MetS by WBV, erythrocyte aggregation and erythrocyte deformability were still significant. Hence, these findings suggest that the microcirculatory defect in MetS is beyond the effect of DM alone, and that alterations in haemorheology are more closely associated with MetS than macrovascular circulation abnormalities. Peripheral artery disease of MetS is more likely due to DM, whereas alteration in haemorheology seems to depend more upon the complex pathophysiology underlying MetS. Czernichow et al. (2010) reported the occurrence of microvascular dysfunction (lower functional capillary density) in MetS participants. The stronger association of MetS with haemorheological parameters rather than peripheral vascular diseases markers may be due to a synergistic effect of different abnormalities or abnormal components underlying MetS. The findings of the present study suggest that an assessment of microvascular abnormalities is equally as important as an assessment of macrovascular functions in MetS.
The values of the odds ratio obtained in the regression analysis depend on the range of data and the scaling. The regression coefficient represents the expected change in y (MetS/non-MetS) for a one unit change in x (the predictor: markers); hence, the magnitude of that coefficient is partly determined by the magnitude of the units used. Therefore, an ROC curve was used to compare the association of different markers with MetS to confirm the outputs of the logistic regression analysis. ROC curves show the diagnostic performance of a test, or the accuracy of a test to discriminate two groups (Hanley & McNeil, 1982) (MetS from non-MetS groups in this case), and the area under the ROC curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups (MetS/non-MetS) (Hanley & McNeil, 1982). ROC curve analysis demonstrated that all of the haemorheological components significantly classified MetS participants (P-values for all curves were <0.0005). The AUC for hsCRP for predicting MetS in the present study was 66.1 %, which is similar to the previously reported values of 68.4% (Han et al., 2002) and 70% (Stefanska, Sypniewska, Błaszkiewicz, Ponikowska, & Cwiklinska-Jurkowska, 2011).

The AUC among the novel cardiovascular risk factors (oxidative stress and chronic inflammation), haemorheological components and peripheral vascular diseases markers were compared for accurate classification of MetS. The AUC for erythrocyte aggregation (critical stress) (0.818; 95% CI: 0.715 to 0.922) was higher than that of the inflammatory marker, hsCRP, and the oxidative stress marker, isoprostanes (Table 4.34). No studies have reported the ROC curve analysis of haemorheological parameters for the correct prediction of MetS, making it difficult to draw comparisons. However, it has been shown that increased erythrocyte aggregation correctly classified patients with vascular disease (Berliner et al., 2005).
Furthermore, ROC curve analysis in the same study revealed that AUC of erythrocyte aggregation for the correct classification of vascular disease was higher than that of ESR, fibrinogen and hsCRP (Berliner et al., 2005). The present study along with the several previous studies, suggest that erythrocyte aggregation could have some important role in the pathogenesis of vascular diseases.

The ROC curve analysis also showed that two of the three haemorheological parameters (critical stress and $E_{I_{\text{max}}}$) better classified MetS than TBPI. The observed superiority of the haemorheological parameters over the peripheral arterial disease markers in predicting MetS further emphasises the importance that should be given to rheological changes occurring in MetS along with vascular assessment. The present study also suggests that rheological changes may occur earlier or more frequently than the peripheral vasculopathy in MetS and early identification of such changes may possibly provide clinical benefits to MetS patients. Similarly, it has been shown that although conventional cardiovascular risk parameters such as triglyceride, HDL-C, LDL-C, total cholesterol, BMI and fibrinogen did not significantly predict cardiac death, haematocrit/WBV significantly predicted cardiac death ($\text{AUC} = 0.716; P = 0.028$) (Kenyeres et al., 2008). The potential for the haemorheological components to be identified as better cardiovascular risk markers due to their strong association with MetS cannot be precluded from the present findings.
5.8 Clinical significance of the study: altered haemorheology and cardiovascular diseases

CVD is a leading cause of death worldwide (Santulli, 2013; Yusuf, Reddy, Ōunpuu, & Anand, 2001), but unfortunately, only half of these cardiovascular events are explained by the well-established ‘classical risk factors’ (Chockalingam et al., 2000). Recently, the roles of haemorheological factors in several CVDs, such as coronary artery disease and myocardial ischaemia, have been demonstrated (Alexy et al., 2015; Toth et al., 2014). One of the objectives of this study was to investigate whether altered haemorheology is associated with novel cardiovascular risk factors such as chronic inflammation, oxidative stress, PAD markers and activation of coagulation in MetS. From ROC curve and binomial logistic regression analysis, it has been demonstrated that haemorheological parameters were strongly associated with MetS, and that they better classified MetS than novel cardiovascular risk factors such as the inflammatory marker, hsCRP; the oxidative stress marker, urinary isoprostanes; thrombotic marker D-dimer; and the PAD markers, TBPI and ABPI (Tables 4.33 and 4.34).

The present study showed that the PAD marker, TBPI, was predicted by the erythrocyte aggregation parameter, critical stress (Table 4.30). There was also a significant linear decrease in the level of critical stress ($P$ trend 0.005), and significant linear increase in the level of critical time ($P$ trend 0.009) across the quartiles of TBPI (Table 4.31). This indicates that erythrocyte aggregation is increased towards the lower values of TBPI, suggesting that erythrocyte aggregation is associated with lower peripheral circulation. Erythrocyte hyperaggregation (erythrocyte aggregation at highest quartile, $\geq 476.9$ Pa) was significantly associated with all of the MetS components, and erythrocyte aggregation was also associated with the novel cardiovascular risk factors, oxidative stress and chronic inflammation (Tables 4.19 and 4.20). It
has been shown that increased erythrocyte aggregation leads to slow coronary blood flow, resulting in altered microcirculation (Ergun-Cagli et al., 2011). Aggregates could form a thrombus at low shear stress (Stoltz & Donner, 1987). Furthermore, several studies have similarly provided convincing evidence that atherosclerotic and thrombotic states are associated with alterations in the haemorheological profile (Koenig & Ernst, 1992; Ricci et al., 2013). An alteration of blood rheology imposes a greater risk of CVD through changes in central and peripheral haemodynamics (Puniyani, Ajmani, & Kale, 1991).

Likewise, in the present study, the PAD marker, TBPI, was also predicted by increased WBV, and there was a significant linear decrease in the value of WBV across the increasing quartiles of TBPI ($P$ trend <0.0005) (Tables 4.30 and 4.31). This indicates that increased WBV could cause damages to the peripheral vascular system, possibly leading to vasculopathy. Stomatocytes number was positively correlated with WBV in the present study. Increased erythrocyte aggregation, and an increase in the prevalence of abnormal shaped erythrocytes (as seen in the present study) may lead to increased WBV (Chien et al. (1967)), possibly reducing microcirculatory flow and increasing the risk of CVD. MetS participants with increased WBV may be at greater risk of suffering from CVD due to the direct effect of WBV on haemodynamics. Previous studies have shown the possible effects of altered haemorheology in microcirculation. Blood pressure is raised following increased WBV (London, 1997), and this possibly increases peripheral vascular resistance. Also, there is a possibility that the pumping requirement of the heart is increased due to the increase in peripheral resistance (Devereux et al., 2000), further increasing the risk of CVD (Kensey, 2003). The heart rate might also increase to compensate for the diminished oxygen supply to tissues, which results from increased
erythrocyte aggregation (Connes et al., 2008). The observed correlation between WBV and CVD risk factors (oxidative stress, chronic inflammation, D-dimer and TBPI) is in agreement with the findings of Presti et al. (2002). They reported a significant positive correlation between homocysteine level, another important CVD risk marker, and increased WBV (Presti et al. 2002). In the Edinburgh Artery Study (prospective study of a random population sample), WBV was shown to be associated with cardiovascular complications (ischemic heart disease and stroke) (Lowe, Lee, Rumley, Price, & Fowkes, 1997). The relative risk of cardiovascular events for WBV was as strong as that of established risk factors such as LDL-C and blood pressure (Lowe et al., 1997).

Increased oxidative stress and chronic inflammation generated in MetS leads to changes in erythrocyte morphology. In the current study, acanthocytes number correlated with oxidative stress markers as well as with hsCRP level (Table 4.23). Wautier et al. (1981) reported increased adhesion of diabetic erythrocytes to the endothelium and positively correlated the extent of adhesion with vascular complications. The changes in morphology of erythrocytes in MetS could be responsible for their adhesion to the endothelium and endothelial activation, and thus lead to vascular complications. This study showed that number of acanthocytes positively correlated with erythrocyte aggregation parameters and negatively correlated with erythrocyte deformability parameters (Table 4.22), indicating a decreased tendency of morphologically altered cells to deform. The increase in erythrocyte aggregation in the present study may be due to abnormal plasmatic factors (autologous plasma was used as a suspending medium for measuring erythrocyte aggregation) or due to a modified cellular properties (aggregability) (Baskurt & Meiselman, 2009) as a pathologic effect of MetS, oxidative stress and inflammation.
Unfavourable haemorheology is associated with activated coagulation system (direction of association not fully known). There was a linear increase in the level of D-dimer across the quartiles of critical stress ($P$ trend= 0.002) and WBV ($P$ trend= 0.049) (refer to 4.5.3). An increased concentration of D-dimer has been associated with coronary heart diseases (Akgul & Uyarel, 2013; Lowe & Rumley, 1999). Rheologically altered erythrocytes could have triggered inappropriate endothelial stimulus leading to inappropriate endothelial secretion of vasoconstrictors, proaggregants, and procoagulant factors (Münzel, Sinning, Post, Warnholtz, & Schulz, 2008), that could further alter the haemorheology (Forconi & Gori, 2013). Another plausible mechanism suggested by studies demonstrating an effect by free radicals on fibrinogen molecules, is that a shift in the oxidant/antioxidant dynamic balance could lead to increased in-vivo oxidation of the fibrinogen molecule, promoting its conversion to fibrin and ultimately D-dimer (Becatti et al., 2014; Martinez, Weisel, & Ischiropoulos, 2013).

This study proposes oxidative stress and chronic inflammation as causes of alterations in haemorheology. An alteration in haemorheology was also associated with increased D-dimer and the peripheral vascular atherosclerotic marker, TBPI. The possible effect of hemorheological factors in cardiovascular medicine has begun to receive more attention (Baskurt & Meiselman, 2003; Popel & Johnson, 2005; Toth et al., 2014). The findings of this study suggest that the negative effect of inflammatory and oxidant molecules in the cardiovascular system could partly be due to the resulting altered haemorheology.
Figure 5.2 (Gyawali, Richards, Hughes, et al., 2014) Altered hemorheology is the bridge that links MetS with cardiovascular complications. Oxidative stress and chronic inflammation decreases erythrocyte deformability and alter erythrocyte morphology. Decrease deforming capacity and altered morphology increase erythrocyte tendency to aggregate. Decrease erythrocyte deformation, increase erythrocyte aggregation and altered erythrocyte morphology increases WBV. Erythrocyte aggregates and morphologically altered erythrocyte could interact with the endothelium causing endothelial and coagulation system activation. Erythrocyte aggregates causes vascular occlusion and blood slow flow as seen from increased TBPI. Altered hemorheology and endothelial activation leads to cardiovascular complications.
Conclusions

The present study investigated the effect of oxidative stress and chronic inflammation on haemorheological parameters (erythrocyte morphology, erythrocyte aggregation, erythrocyte deformability and WBV) in patients with and without MetS. Also, the alterations in the haemorheological parameters were associated with cardiovascular risk factors, D-dimer and peripheral vascular diseases markers (ABPI and TBPI). Several important conclusions can be drawn from the findings.

Increased concentrations of urinary isoprostanes and decreased concentrations of erythrocyte SOD and erythrocyte GSH were observed among MetS patients when compared to healthy controls. Similarly, hsCRP level was found to be higher in MetS patients when compared to healthy controls. From these findings, it can be concluded that oxidative stress and low grade inflammation is higher in MetS group when compared to healthy controls.

Similarly, haemorheological parameters such as WBV and erythrocyte aggregation level were higher, whereas erythrocyte deformability ($E_{\text{max}}$) was lower, in participants with MetS when compared to participants without MetS. Abnormal erythrocyte morphology (acanthocytes, echinocytes and stomatocytes) were more prevalent and biconcave shape erythrocytes was less prevalent in MetS group compared to healthy controls. These findings suggest that haemorheology is grossly altered in MetS. MetS was associated with the highest quartile of WBV and erythrocyte aggregation, and lowest quartile of $E_{\text{max}}$. The findings of the association of MetS with the unfavourable rheological quartile suggest that altered haemorheology may have a role in determining the complications that could arise from MetS.
The increased oxidative stress and inflammation were correlated with altered haemorheology in MetS in the present study. Urinary isoprostanes and hsCRP were negatively correlated with biconcave cells, and positively correlated with acanthocytes. On the contrary, GSH was positively correlated with biconcave cells and negatively correlated with acanthocytes. This indicates that increased oxidative stress and chronic inflammation in MetS negatively affect erythrocyte morphology. Erythrocytes change shape because of continuous oxidative damage to membrane proteins and lipids. Altered erythrocyte morphology in turn was correlated with abnormal erythrocyte deformability and erythrocyte aggregation. A negative correlation of acanthocytes was seen with erythrocyte deformability, and a positive correlation was seen with erythrocyte aggregation in the present study, suggesting that morphological changes affect other rheological characteristics of cells. An increased generation of inflammatory molecules in MetS increases adhesive proteins in the milieu of erythrocytes and these inflammatory molecules may further provide a suitable environment for morphologically abnormal cells to aggregate. Overall, it can be concluded that oxidative stress and chronic inflammation generated in the state of MetS affect haemorheological parameters. The interplay of oxidative stress, inflammation and altered haemorheology potentially induces cardiovascular diseases by slowing down the rate of blood flow through capillaries, possibly leading to endothelial activation, decreased oxygen delivery and ultimately causing vasculopathy. The relative risk of cardiovascular events for WBV has been reported to be as strong as established risk factors, such as LDL cholesterol and blood pressure (Lowe et al., 1997). The negative effect of chronic inflammation and oxidative stress in the cardiovascular system could partly be due to the resulting altered haemorheology.
Blood flow in the peripheral vascular system was assessed by the measurement of ABPI and TBPI. TBPI was reduced among MetS group compared with healthy controls. This indicates that peripheral blood flow in small blood vessels is reduced in MetS. The decrease in TBPI positively correlated with the levels of erythrocyte GSH and SOD. Similarly, oxidative stress (reduced SOD and GSH and increased isoprostanes) predicted the value of TBPI. This suggests that a decrease in blood flow is associated with the generation of oxidative stress. The findings of the present study underscore the potential mechanism of oxidative stress-driven progression of peripheral atherosclerotic disease. TBPI value was predicted by erythrocyte aggregation and WBV. There was a linear decrease in the level of critical stress and a linear increase in the level of critical time across the quartiles of TBPI. Similarly, there was a linear decrease in the value of WBV across the increasing quartiles of TBPI. This finding suggests that unfavorable rheology is associated with decreased blood flow in vessels. It also further reinforces the previous findings that oxidative stress and chronic inflammation lead to abnormal erythrocyte morphology and alterations in other blood rheological properties, which may subsequently decrease blood flow in capillaries and ultimately cause vasculopathy.

None of the MetS components predicted hyperviscosity or reduced erythrocyte deformability, but all of the rheological components (erythrocyte aggregation, erythrocyte deformability, WBV) predicted MetS even after they were adjusted for the effect of age, sex and presence of DM. This suggests that the synergistic effect of the combination of abnormalities in MetS is more severe and significant than the effect of individual MetS components. These findings emphasise the significance of MetS and ascertain that outcomes of a combination of the three components of MetS are more severe than that of the components on their own. Haemorheological and
circulation defects were present in MetS (as evidenced by a reduced TBPI). However, binomial logistic regression analysis and ROC curve analysis both showed that haemorheological parameters were better associated with MetS than were oxidative stress, chronic inflammation and peripheral vasculopathy. The stronger association of MetS with haemorheological parameters strongly supports the prediction that haemorheological parameters are better cardiovascular risk factor markers than oxidative stress and chronic inflammation. Similarly, the stronger association of MetS with haemorheological parameters than peripheral vascular diseases markers suggests that rheological changes may occur earlier or more frequently than peripheral vasculopathy in MetS. Unfavourable changes in haemorheological parameters are more likely to affect small blood vessels; hence, an assessment of microvascular abnormalities is equally as important as an assessment of macrovascular blood flow in MetS. Altered haemorheology could be the bridge that connects MetS with increased CVD, and a defect in microcirculation due to altered haemorheology could be one of the several pathogenic mechanisms underlying MetS.
Recommendations

The present study suggests that the oxidative stress and inflammation is increased in the state of MetS. The increased oxidative stress and inflammation affected haemorheological parameters in the study. The complex interplay of oxidative stress, inflammation and altered haemorheology are strong cardiovascular risk factors, and thus, this study recommends that assessment of haemorheological parameters along with other novel cardiovascular risk factors would add value in assessment of cardiovascular risk. The IDF MetS definition working group has acknowledged that the best definition of MetS is yet to come (Alberti et al., 2006). MetS has a haemodynamic basis and specific studies in this direction would make a significant contribution towards developing an updated definition of MetS. The effect of MetS is more severe than the effect of its individual components in enhancing oxidative stress, inflammation and altering haemorheology. The ROC curve analysis and the regression analysis demonstrated that erythrocyte aggregation is superior to oxidative stress markers and chronic inflammatory markers for accurate prediction of MetS. Also, since erythrocyte aggregation is significantly associated with oxidative stress and chronic inflammation generated in MetS, it could be included as a component of MetS. The establishment of haemorheology as a component of MetS would no doubt be an invaluable risk marker for CVD.

This study also suggests that haemorheological defects occur more frequently and possibly earlier than vasculopathy. Therefore, it is recommended that the assessment of haemorheological defects may provide some clinical advantages in early decision making among MetS patients. The present study has demonstrated reduced TBPI, and thus, reduced peripheral blood flow in MetS patients. Reduced TBPI was strongly associated with oxidative stress haemorheological
components. Hence, the present study recommends the assessment of small blood vessels and capillary blood circulation in MetS patients along with the assessment of large vessels functions.

References


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20 August 2012

Mr Prajwal Gyawali
School of Community Health
ALBURY-WODONGA CAMPUS

Dear Mr Gyawali,

Your research proposal entitled "Oxidative stress and haemorheology in subjects with metabolic syndrome: diabetes mellitus, hypertension, obesity" has been reviewed by Charles Sturt University’s (CSU) Human Research Ethics Committee (HREC).

The CSU HREC operates in accordance with the National Health and Medical Research Council’s National Statement on Ethical Conduct in Research Involving Humans.

I am pleased to advise that the project meets the requirements of the National Statement; and ethical approval for this research is granted for a twelve month period from 20/8/12.

The protocol number issued with respect to this project is 2012/131. Please be sure to quote this number when responding to any request made by the Committee.

Please note the following conditions of approval:

• all Consent Forms and Information Sheets are to be printed on Charles Sturt University letterhead. Students should liaise with their Supervisor to arrange to have these documents printed;
• you must notify the Committee immediately in writing should your research differ in any way from that proposed. Forms are available at http://www.csu.edu.au/
data/assets/word_doc/0010/176833/ehrec_annrep.doc
• you must notify the Committee immediately if any serious and or unexpected adverse events or outcomes occur associated with your research, that might affect the participants and therefore ethical acceptability of the project. An Adverse Incident form is available from the website; as above;
• amendments to the research design must be reviewed and approved by the Human Research Ethics Committee before commencement. Forms are available at the website above;

Version 2

PRA

www.csu.edu.au
• if an extension of the approval period is required, a request must be submitted to the Human Research Ethics Committee. Forms are available at the website above;
• you are required to complete a Progress Report form, which can be downloaded as above, by 20/8/13 if your research has not been completed by that date;
• you are required to submit a final report, the form is available from the website above.

YOU ARE REMINDED THAT AN APPROVAL LETTER FROM THE CSU HREC CONSTITUTES ETHICAL APPROVAL ONLY.

If your research involves the use of radiation, biological materials, chemicals or animals a separate approval is required from the appropriate University Committee.

The Committee wishes you well in your research and please do not hesitate to contact the Executive Officer on telephone (02) 6338 4628 or email ethics@csu.edu.au if you have any enquiries.

Yours sincerely

Julie Hicks
Executive Officer
Human Research Ethics Committee
Direct Telephone: (02) 6338 4628
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Information sheet

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Research Project Participant Information Sheet

Title: Oxidative stress and haemorheology in subjects with metabolic syndrome: diabetes mellitus, hypertension, obesity.

You are invited to participate in the study of the red blood cells abnormalities in diabetes mellitus, cardiovascular diseases and obesity. Pregnant women, children with age less than 18 years, people with any form of mental diseases, Asian and Indian population (Europeans will be considered) and non-ambulatory patients (admitted in hospital) will not be considered in the study.

Patients with diabetes mellitus and hypertension will be included in the experimental group. All other volunteer participants who do not have diabetes and hypertension will be included in the control group. This study is being conducted by PhD student Mr. Prajwal Gyawali under the supervision of Dr. Ross Richards, Dr. P Tinley, Dr. P Bwititi and Dr. EU Nwose.

Cardiovascular diseases, Diabetes mellitus and obesity are a common medical condition. There is an alteration in the red blood cells of our body in these diseases state and this abnormality in our red blood cells may further complicate the disease progression. The study aims to measure specific changes that occur in erythrocytes’ shape, flexibility and anti-oxidant capacity. The findings of the study may help in the assessment of disease, its severity and progression. With further research, the data may also contribute to a better understanding of the possible causes of diseases.

If you decide to participate in the research, 20 mL of blood will be obtained from you by venipuncture. The procedure of blood collection is absolutely safe and it takes maximum of five minutes. Approximately 10mL of mid-stream urine sample will be collected in the privacy of the nearby disabled access toilet. The blood and urine sample obtained from you will be processed in the university laboratory to obtain the results.

You will be requested to answer some questions related to your medical problem, dietary habit and medications. It takes about 10 minutes. It is your choice whether you answer or not any particular question/questions. Your blood pressure will be recorded while sitting. Blood pressure will also be
recorded from your toe. Also your waist circumference, height and weight will be measured. The information given by you and the results obtained from your blood and urine samples will be used to obtain findings of the research and the conclusion drawn from the findings will be published in several scientific journals.

Mr. Prajwal Gyawali would also like to contact your general practitioner or medical specialist to view your medical history. Only medical details that are relevant to your diagnosis will be requested. This information will be kept strictly confidential and only used to verify diagnosis and/or disease complications according to standard clinical criteria.

Your name will not be disclosed in any publications and will be kept confidential. Your name will not be used to identify your blood and urine samples or on your information sheet or questionnaire. A unique code will be given to them before analysis instead of using your name. The blood and urine sample and information provided by you will be handled only by me and my supervisor and all the information given will be kept safely in a locked cabinet at the university.

Your participation is entirely voluntary and you can withdraw at any time with no consequences to yourself. You can also request that your blood and urine sample and data be destroyed. There will be no direct benefit to you from participating, however this study will contribute to knowledge of the role of oxidative stress and haemorheology in diabetes mellitus, hypertension and obesity.

**NOTE:** Charles Sturt University’s Human Research Ethics Committee has approved this project. If you have any complaints or reservations about the ethical conduct of this project, you may contact the Committee through the Executive Officer:

<table>
<thead>
<tr>
<th>The Executive Officer</th>
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<tbody>
<tr>
<td>Human Research Ethics Committee</td>
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<tr>
<td>Office of Academic Governance</td>
</tr>
<tr>
<td>Charles Sturt University</td>
</tr>
<tr>
<td>Panorama Avenue</td>
</tr>
<tr>
<td>Bathurst NSW 2795</td>
</tr>
</tbody>
</table>

Tel: (02) 6338 4628  
Email: ethics@csu.edu.au

Any issues you raise will be treated in confidence and investigated fully and you will be informed of the outcome.
Questionnaire

Title: Oxidative stress and haemorheology in subjects with metabolic syndrome: diabetes mellitus, hypertension, obesity

The following questionnaire is designed to find out some information about your medical history in relation to the study. This information is required so the data can be comprehensively analysed. Your confidentiality will be maintained at all times.

Date:

Identification (for investigator use)

Surname:
Name:
Medical Practitioners name/address:

Male/ female Age: .......................   DOB:............................

• Have you been diagnosed with Diabetes?....................
  Type I (     ) or Type II (     )
• Number of years diabetic......................
• Have you been diagnosed with any cardiac problems?
• Number of years cardiac problem....................
• Have you been told by a health professional that you have high blood pressure? Yes ( ) No (     )
• Do you have a family history of Diabetes? Yes ( ) No (     )
• Do you have a family history of hypertension? Yes ( ) No (     )
• Do you smoke? Yes ( ) No (     )
• If yes generally how many cigarettes per day? ..................

• Do you consume alcohol? Yes ( ) No ( )
  o If yes how many glasses (mL) per day?......................

• How much exercise would you participate in, in a typical day?
  o No exercise or less than 15 minutes
  o Half hour
  o 1 hour
  o 2 hour
  o 3 hour
  o > 3 hour

• What are your current prescription medications? (please include all the medications you are taking)

• Have you had/have any of the following?
  o Thyroid problem ( )
  o Kidney problem ( )
  o Hypertension during past pregnancies ( )
  o Rheumatic fever ( )