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Is Darkfield Microscopic Examination of Fresh Capillary Blood a Valid Point of Care Screening Tool to Assess Metabolic Syndrome Risk Factors?

Katrina Joanne Reeve
BA, BHSc (Naturopathy), Grad Dip Opera, LTCL

This thesis is presented in fulfilment of the requirement for the degree of Master of Science at Southern Cross University

March 2015

Declaration

I certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

I acknowledge that I have read and understood the University's rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. I certify that I have complied with the rules, requirements, procedures and policy of the University.

Name:_____

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Abstract

Is Darkfield Microscopic Examination of Fresh Capillary Blood a Valid Point of Care Screening Tool to Assess Metabolic Syndrome Risk Factors?

Fresh capillary blood analysis using darkfield microscopy (FCB-DM) is a point of care screening tool used by complementary healthcare practitioners in Australia, where capillary blood cells are untreated and viewed immediately. The aim of the study was to determine the validity of FCB-DM by examining the relationship between FCB-DM variables and venous blood pathology tests when screening for metabolic syndrome risk factors.

Selected markers of metabolic syndrome such as blood lipids, inflammatory markers and the differential white blood cell count were investigated. A null hypothesis was tested, that FCB-DM results would not correlate to venous blood pathology results.

The project was divided into two studies: the first study was on metabolic syndrome risk factors where participants were specifically recruited for the project in Brisbane (n=51) and Lismore (n=19). The second was a study of differential white blood cell counts where data from the above study were combined with retrospective data gathered from files at a naturopathic clinic (n=125).

In the Brisbane cohort data, we found moderate but significant correlations between FCB-DM fasting platelet area and haematology platelet counts (r_s (50) = .364, p < .05) and fasting platelet area and C-reactive protein (r (50) = .436, p < .05). Correlations were also between fasting FCB-DM chylomicron remnant counts and hematology triglycerides (r_s (50) = .298, p < .05), fasting insulin (r (50) = .334, p < .05), HbA1c (r_s (51) = .353, p < .05), $HOMA_B$ (r (47) = .309, p < .05) and $HOMA_{IR}$ (r (46) = .305, p < .05).

In the Lismore data the size of the cohort caused a type two error for most parameters, although fasting average platelet number was found to be significantly correlated with the pathology measure of platelets (r (19) = .470 p <.05).

The differential white blood cell counts determined for each participant by the two techniques were compared in the second study. Despite some significant differences in some of the mean

scores, significant and positive correlations were found between the two techniques in neutrophil (t (124) = -9.24, $p < .05$; $r = .60$, $p < .05$), lymphocyte (t (124) = 8.56, $p < .05$; $r = .63$, $p < .05$), monocyte (t (124) = 1.25, $p < .05$; $r_s = .32$, $p < .05$) and eosinophil (t (124) = 1.27, $p > .05$; $r_s = .596$, $p < .05$) cell populations.

This research provided new evidence that supports FCB-DM as a potential point of care screening tool for metabolic risk factors based on moderate levels of correlations.

Presentations and Publications

Original journal paper

Reeve, K., Arellano, J., W, G. T., Reilly, W., Smith, B., & Zhou, S. (2015). A comparison of differential leucocyte counts measured by conventional automated venous haematology and darkfield microscopic examination of fresh capillary blood. Manuscript accepted for publication in Advances in Integrative Medicine (doi:10.1016/j.aimed.2015.05.001) (Appendix A, Abstract).

Conference presentations

Reeve, K. (2014). *Is darkfield microscopic analysis of fresh capillary blood a viable alternative option to venous haematology for health screening?* Paper presented at the Australian Health and Medical Research Congress, Melbourne. (Appendix B, Abstract)

Reeve, K., & Reilly, W. (2011). *Identification of abnormal lymphocytes during routine screening using HemaviewTM – a case study*. Poster presented at the Annual Molecular and Experimental Pathology Society of Australia Conference, Brisbane. (Appendix C, Poster)

Reeve, K. (2011). *HemaviewTM: A Clinically Useful Tool for White Blood Cell Differential Counts.* Paper presented at the Annual Molecular and Experimental Pathology Society of Australia Conference. (Appendix D, Abstract).

Invited plenary speaker at the International Congress on Natural Medicine, *Clinical validation of Hemaview^{TM} live blood screening and its application to cardiometabolic risk assessment*. Melbourne, June 2015.

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To my Mum, the most amazing and inspiring woman I have had the pleasure to know. Her belief in my abilities never wavered and her scientific expertise has been invaluable. Who'd have thought your opera singer daughter would have found herself here? And lastly, my darling, patient, supportive and steadfast husband who rode the winding path of this roller coaster with me. You are my rock.

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This work is dedicated to the memory of the esteemed Dr Tini Grüner, who I hope will be proud, and know that we *finally* got there.

Table of Contents

1 Introduction 1
1.1 Statement of Problem and Research Questions 4
1.2 Aim and Hypotheses 5
1.3 Significance 6
2 Literature Review 7
2.1 A History of Darkfield Microscopy 7
2.1.1 Darkfield microscopy in dentistry. 8
2.1.2 Darkfield microscopy in microbiology. 9
2.2 Fresh Capillary Blood Analysis 10
2.2.1 Günther Enderlein. 10
2.2.2 Philip Hoekstra. 12
2.2.3 Current literature and research. 13
2.2.4 Point of care screening. 14
2.3 Pathology Techniques 15
2.3.1 Blood films. 15
2.3.2 Automated blood analysers. 17
2.4 Capillary and Venous Blood Compared 19
2.5 Determining the Validity of FCB-DM 22
2.6 The Global Epidemic of Metabolic Syndrome and Obesity 23
2.6.1 Epidemiology 26
2.6.2 The rising cost of obesity. 27
2.6.3 Obesity, metabolic syndrome and insulin resistance. 27
2.6.4 Obesity, metabolic syndrome and inflammation. 28
2.6.5 Obesity, metabolic syndrome and hyperlipidaemia. 29
2.6.6 Anthropometric measurements. 30
2.6.6.1 Homeostatic model assessment. 32
2.7 Clinical Potential for FCB-DM 33
2.8 Fresh capillary blood analysis variables in obesity. 33
2.8.1 Platelet aggregation. 34
2.8.2 Fibrin. 35
2.8.3 Chylomicron remnant clearance. 38
2.8.4 Differential white blood cell counts. 40
2.8.4.1 Neutrophils. 41
2.8.4.2 Lymphocytes. 43
2.8.4.3 Monocytes. 46
2.8.4.4 Eosinophils. 47
2.8.4.5 Basophils. 49
2.8.4.6 Differential white blood cell count. 49
2.8.4.7 Differential white blood cell counts – manual v's automated. 50
2.9 Summary 50
3 Methodology 52

3.1 Metabolic Syndrome Study....52
3.1.1 Target population, sample selection and size. 52
3.1.2 Participant recruitment.....53
3.1.3 Inclusion and exclusion criteria. 53
3.1.4 Study design.....54
3.1.5 Blinding.....56
3.1.5.1 Blinding – Brisbane. 56
3.1.5.2 Blinding – Lismore. 57
3.1.6 Blood collection procedure – pathology. 57
3.1.7 Blood collection procedure - fresh capillary blood analysis.....58
3.1.8 Fresh capillary blood analytical technique. 59
3.1.9 Microscope image calibration.....61
3.1.10 Data collection. 61
3.1.11 Statistical analysis.....61
3.1.12 Intra-measurer reliability. 63
3.1.13 Limitations and delimitations. 63
3.1.14 Ethics approval.....63
3.2 Differential White Blood Cell Study....64
3.2.1 Study design.....64
3.2.2 Inclusion and exclusion criteria. 64
3.2.3 Blinding.....65
3.2.4 Procedures.....65
3.2.5 Data analysis. 65
3.2.6 Ethics approval.....66

4 Results 67
4.1 Metabolic Syndrome Study....67
4.1.1 Characteristics of the participants.....67
4.1.2 Data collection site differences.....67
4.1.3 Platelets – Brisbane.....68
4.1.4 Platelets – Lismore.....69
4.1.5 Fibrin – Brisbane.....69
4.1.6 Fibrin – Lismore. 70
4.1.7 Chylomicron remnants – Brisbane.....70
4.1.7.1 Correlations.....70
4.1.7.2 Influence of BMI.....71
4.1.8 Chylomicron remnants – Lismore. 71
4.1.9 Reliability of analysis. 72
4.2 Differential White Blood Cell Counts....73
4.2.1 Correlation. 73

5 Discussion....75
5.1 Metabolic Syndrome Study....75
5.1.1 Platelets.....76
5.1.2 Fibrin.....77

5.1.3 Chylomicron remnants. 78
5.2 Differential White Blood Cell Study 80
5.3 Methodological Limitations in the Metabolic Syndrome Study 83
5.4 Methodological Limitations in the Differential White Blood Cell Study 85
5.5 Future Study Directions 85

6 Conclusions 87

7 Appendices 110
7.1 Appendix A. Published Paper. 110
7.2 Appendix B. Abstract, Oral Presentation, AHMRC 2014. 111
7.3 Appendix C. Poster, MEPSA 2013. 112
7.4 Appendix D. Abstract, Oral Presentation, MEPSA 2011. 113
7.5 Appendix E. Waist Circumference Thresholds. 114
7.6 Appendix F. Causes of Neutropenia and Neutrophilia. 115
7.7 Appendix G. Recruitment Letter. 116
7.8 Appendix H. Southern Cross University Recruitment Email. 117
7.9 Appendix I. Raw Data Table – Fibrin and Platelets. 118
7.10 Appendix J. Raw Data Table – Chylomicron Remnants. 120
7.11 Appendix K. Ethics approvals. 122
7.12 Appendix L. – Correlation results 124

List of Figures

Figure 1. A schematic configuration of conventional darkfield microscopy. 8
Figure 2. A schematic presentation of the progression and development of metabolic syndrome. 23
Figure 3. Increasing BMI in Australia. 26
Figure 4. Electron micrograph images of platelets. 34
Figure 5. Platelet clusters surrounded by erythrocytes as seen by FCB-DM. 36
Figure 6. Fibrin cluster as seen by FCB-DM. 37
Figure 7. Diffuse fibrin as seen by FCB-DM. 37
Figure 8. Chylomicron remnants as seen by FCB-DM. 39
Figure 9. Neutrophils as seen by FCB-DM. 42
Figure 10. Monocyte and lymphocyte as seen by FCB-DM. 45
Figure 11. Neutrophil and eosinophil as seen by FCB-DM. 48
Figure 12. FCB-DM slide territories. 59
Figure 13. The direction of movement across the blood film. 60
Figure 14. Chylomicron remnants and BMI. 72
Figure 15. Differential white blood cell scatter plots. 74
Figure16. Comparison of data images between Brisbane (a) and Lismore (b). 83

List of Tables

Table 1. Clinical indications for blood smear analysis request. 16
Table 2. Criteria for clinical diagnosis of metabolic syndrome. 25
Table 3. International classification of BMI. 31
Table 4. FCB-DM and venous pathology tests comparative variables. 55
Table 5. Participants' general characteristics mean values. 67
Table 6. Participants' platelets mean values. 68
Table 7. Participants' FCB-DM fibrin and markers of inflammation mean values. 69
Table 8. Participants' FCB-DM chylomicron and lipid measures mean values. 71
Table 9. Differential white blood cell counts. 73

List of Abbreviations

ABS	Australian Bureau of Statistics
BMI	Body mass index
CAM	Complementary and alternative medicine
CIHCC	Coorparoo Integrated Health Care Centre
CFA	Continuous flow analysis
CRP	C-reactive protein
DM	Darkfield microscopy
ESR	Erythrocyte sedimentation rate
FCB-DM	Fresh capillary blood analysis with darkfield microscopy
FIA	Flow injection analysis
HbA1c	Glycosylated haemoglobin
HDL	High density lipoprotein
HOMA	Homeostatic model assessment
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
QML	Queensland Medical Laboratory
SCU	Southern Cross University
TNF	Tumour necrosis factor
TEM	Technical error of the mean
WHO	World Health Organisation

1 Introduction

The use of complementary and alternative medicine (CAM) in Australia has significantly increased over the last 20 years (Lin et al., 2009) with an estimated 69.8% of the Australian population using some form of CAM each year, at an estimated expenditure of AU $4.13 billion per annum (Harris, Cooper, Relton, & Thomas, 2012; Lin et al., 2009; A. H. MacLennan, Wilson, & Taylor, 2002; Xue, Zhang, Lin, Da Costa, & Story, 2007). With this strong growth in the utilisation of CAM comes the increased need for research into the safety, validity and efficacy of CAM practices.

Fresh capillary blood analysis using darkfield microscopy (FCB-DM) is a point of care screening tool used by CAM practitioners in Australia and New Zealand (Bensoussan, Myers, Wu, & O'Connor, 2004). Using darkfield microscopy, blood particles are viewed immediately in their fresh, untreated state, theoretically allowing the practitioner to make an assessment of the patient's blood cell morphology and dynamics (Thorup, 1987).

The practice of FCB-DM is currently not regulated. There are a number of different FCB-DM techniques currently practiced in Australia that differ in both method and interpretation of findings. Developed by Health World Ltd., Hemaview™ is the trade name of the specific live blood analysis method investigated in this study. This specific FCB-DM technique is based on blood film pathology practices and has a five-day training program in Australia. It is used by approximately 7000 registered CAM practitioners in Australia, as a rapid, point of care screening tool to inform clinical practice, educate and motivate patients regarding their health, and improve treatment compliance (Reilly, Mannion, & Barbiellini, 2010; Vitetta et al., 2005). A 2004 survey on the nature of complementary medicine in Australia discovered that 11.8% of practitioners surveyed used live blood analysis in the clinical setting (Bensoussan et al., 2004). Despite this, there is scarce scientific evidence that the observations made with FCB-DM relate to the acknowledged standard venous blood pathology testing.

Notwithstanding the paucity of scientific substantiation, CAM practitioners throughout Australia use FCB-DM in clinical practice as a screening tool. This study sought to provide preliminary data pertaining to the validity of FCB-DM as a screening tool for metabolic syndrome risk, by examining the relationship between FCB-DM data and commercial venous blood pathology results.

Australia has the fifth highest rate of obesity in the world, and it is estimated that 63.4% of adult Australians are overweight or obese (Australian Bureau of Statistics, 2013). Patients with a high body mass index (BMI) or high waist measurement are at significantly increased risk of developing cardiovascular disease, insulin resistance and metabolic syndrome (Keller, 2006) placing a heavy burden on the national healthcare system (Colagiuri et al., 2010). A valid point of care screening tool for metabolic syndrome risk has the potential to ease the healthcare burden and improve patient outcomes through early identification.

A wealth of research has shown that people with metabolic syndrome show evidence of chronic inflammation (Anand et al., 2003; Das, 2002; Dominiczak, 2003b; Eckel, Grundy, & Zimmet, 2005; E. S. Ford, 2003; Haffner, 2006), dysglycaemia (Eckel et al., 2005; S. M. Grundy, Brewer, Cleeman, Smith, & Lenfant, 2004; Samson & Garber, 2014) and alterations to lipid metabolism (Abbott et al., 1987; Chahil & Ginsberg, 2006; Frayn, 1993; Garg, 1996; Kinoshita et al., 2009). As part of the medical diagnosis for metabolic syndrome, medical clinicians routinely request a number of venous blood pathology tests such as platelet counts, eosinophil sedimentation rate (ESR) and C-reactive protein (CRP), blood lipids (including triglycerides, high-density lipoprotein and low-density lipoprotein), fasting glucose and insulin and glycosylated haemoglobin (HbA1c) (K. G. M. M. Alberti, Zimmet, & Shaw, 2006; Anand et al., 2003). Though it has not been scientifically researched till now, clinical observations suggest that a relationship exists between platelet, fibrin and chylomicron remnant presence observed in FCB-DM and metabolic dysfunction (Reilly, 2011, p. 65).

Platelets are non-nucleate cytoplasmic fragments involved in haemostasis, that can be clearly observed in darkfield microscopy (Slayter, 1989). Abdominal obesity has been linked with prothrombotic changes, as adipose tissue releases cytokines and

adipokines, which influence haemostatic and fibrinolytic balance, platelet function and inflammation (Anfossi, Russo, & Trovati, 2009; Russo, 2012).

Low grade chronic inflammation is present in obesity (Gregor & Hotamisligil, 2011; Hotamisligil, 2006; Wellen & Hotamisligil, 2003) where systemic increases in circulating cytokines and acute phase proteins, recruitment of white blood cells to inflamed tissue, and generation of tissue repair responses such as fibrosis is seen (Lumeng & Saltiel, 2011). Correlative and causative links have been demonstrated between low-grade inflammation and insulin resistance (Dandona, Aljada, & Bandyopadhyay, 2004; de Heredia, Gomez-Martinez, & Marcos, 2012; Shoelson, Herrero, & Naaz, 2007; Xu et al., 2003; Zeyda & Stulnig, 2009).

Inflammatory markers in venous blood such as eosinophil sedimentation rate (ESR) and C-reactive protein (CRP) are routinely requested in clinical practice to ascertain the presence of inflammation in patients (Corrado & Novo, 2007). Obesity and a higher BMI are both associated with higher CRP concentrations (Aronson et al., 2004; Visser, Bouter, McQuillan, Wener, & Harris, 1999). Both CRP and fibrin proteins are involved in the acute phase of inflammation and clotting. Fibrin is visible in darkfield microscopic (DM) examination of capillary blood (Barratt, 1920).

Peripheral neutrophil, monocyte and lymphocyte counts have been independently and significantly associated with the cluster of symptoms indicative of metabolic syndrome diagnosis (Kim et al., 2008; J. C. R. Tsai et al., 2007). In an international nutrition workshop in 2009, Dr. Subramanian presented research showing the number of macrophages in adipose tissue strongly correlated with body weight, BMI and total body fat in rodents and humans (Subramanian & Ferrante, 2009). Macrophages have been identified as the primary source of high levels of inflammatory cytokines present in obesity and it has been theorised that macrophage infiltration into adipose tissue is associated with inhibited adipocyte differentiation, hypertrophy, altered secretion of adipokines and increased storage of lipid within liver, muscle and other non-adipose tissues (Heilbronn & Campbell, 2008). These changes are partially responsible for the hyperlipidaemic state seen in obesity.

Chylomicrons are lipoprotein particles that transport lipids from enterocytes

into the body via the intestinal lymph and systemic circulation (Gropper, Smith, & Groff, 2005; Silbernagel & Despopoulos, 2009). They consist mainly of triglycerides, phospholipids and cholesterol. What remains in the blood after lipoprotein lipase removes part of the triglyceride from the chylomicron, is termed a chylomicron remnant (Gropper, Smith, & Groff, 2005). Patients with impaired lipid metabolism may demonstrate higher concentrations of chylomicron remnant material in fasting capillary blood (Vitetta et al., 2005), and indeed slower clearance of chylous material postprandially. Furthermore, there is increasing evidence in the literature that insulin resistance is associated with postprandial hyperlipidaemia (Dominiczak, 2003b; Eleftheriadou, Grigoropoulou, Katsilambros, & Tentolouris, 2008; Esposito et al., 2004; Ferrannini, Haffner, Mitchell, & Stern, 1991; Kinoshita et al., 2009), characterised by raised numbers of chylomicron remnants (Kinoshita et al., 2009).

Thus it can be seen that metabolic syndrome encompasses a group of clinical findings including abdominal obesity, high blood glucose levels, high triglyceride levels, low high-density lipoprotein (HDL) levels (Dominiczak, 2003a; S. M. Grundy et al., 2004; Samson & Garber, 2014) and chronic, low grade inflammation (Das, 2002; E. S. Ford, 2003; Shoelson et al., 2007). This collection of clinical findings makes this group of patients the ideal cohort to examine the validity of FCB-DM. Concurrent validity measures how well a particular test correlates with a previously validated measure (George, Batterham, & Sullivan, 2003; Hurley, Denegar, & Hertel, 2011; Salkind, 2012). Validity, in this context, refers to the strength of association, or correlation, between FCB-DM measures of specific variables in comparison to the relevant acknowledged standard venous blood pathology test measures.

1.1 Statement of Problem and Research Questions

The paucity of rigorous scientific research into the validity of FCB-DM throws question over its use as a screening tool in health care. This study aims to investigate the validity of FCB-DM in screening for metabolic syndrome risk factors in two projects:

1. Metabolic syndrome study, where the relationship between FCB-DM variables and venous blood pathology measures were examined.

2. Differential white blood cell study, where correlations between FCB-DM and venous blood pathology differential white blood cell counts were examined.

1.2 Aim and Hypotheses

The aim of the study was to determine the validity of FCB-DM by examining the relationship between FCB-DM variables and venous blood pathology tests when screening for metabolic syndrome risk factors.

This thesis investigated if relationships existed between the FCB-DM variables of platelet number and/or platelet area with venous blood pathology platelet counts; FCB-DM fibrin number and/or fibrin area with ESR and CRP; and FCB-DM chylomicron remnant presence with triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein (LDL), fasting glucose and insulin and glycosylated haemoglobin (HbA1c).

A null hypothesis was tested, that FCB-DM results would not demonstrate a significant relationship to venous blood pathology results in the following measures:

A. The platelet area and number measured in fasting FCB-DM would not correlate to the platelet counts in fasting venous blood measured by conventional pathology tests.

B. The presence of fibrin measured in fasting FCB-DM would not correlate to ESR and CRP in fasting venous blood measured by conventional pathology tests; and there would be no correlation between BMI and FCB-DM fibrin measures.

C. The presence of chylomicron remnants in fasting FCB-DM would not correlate to the lipid profile, glucose, insulin and HbA1c in fasting venous blood measured by conventional pathology tests; and there would be no difference in chylomicron remnant counts between the fasting and postprandial samples as measured by FCB-DM.

D. The differential white blood cell counts as measured by FCB-DM would be different to, and not correlate with, that in the venous blood measured by conventional pathology tests.

1.3 Significance

Because the FCB-DM technique is utilised in CAM practices throughout Australia, understanding the clinical usefulness of the information it can supply is important in providing safe and effective treatments for patients. If found to be valid, the less invasive nature of FCB-DM in comparison with venous blood extraction, lends the technique to many health care applications, ancillary to the acknowledged standard venous blood pathology tests.

The results of this study will help health care practitioners make a more informed choice about the clinical application of FCB-DM as a screening tool for metabolic syndrome risk factors.

2 Literature Review

This narrative literature review provides analysis of the current literature available to the author in the following areas:

- Darkfield microscopy and fresh capillary blood analysis
- Venous blood pathology tests
- Venous and capillary blood variables compared
- Metabolic syndrome
- Differential white blood cell counts

A search of the literature was conducted by searching Medline, Pubmed and Embase.com databases using the keywords, phrases and combinations of: 'Hemaview', 'darkfield microscopy', 'darkfield microscope', 'dark field microscopy', 'dark ground microscopy'', 'Günther Enderlein', 'blood counts', 'differential counts', 'differential white blood cell counts', 'white blood cells', 'leukocyte count', 'metabolic syndrome', 'inflammation', 'blood glucose', 'obesity', 'platelet', 'fibrin', 'clotting cascade', 'HOMA', 'chylomicron', 'chylomicron remnant', 'blood lipids'. A total of 1205 articles were collected, 328 were cited and referenced.

2.1 A History of Darkfield Microscopy

Joseph Jackson Lister is credited as the creator of the first darkfield microscope in Germany in 1830 (Simon Henry, 1920; Teut, Lüdtke, & Warning, 2006). By 1909, the Bausch and Lomb Company were manufacturing darkfield microscopes to assist chemists studying colloidal reactions such as the setting of cement. Central light rays along the optical axis of the microscope, that usually surround the specimen, are blocked out in darkfield microscopes, thus the sample is illuminated with oblique light from large angles. A seven-sided toroidal mirror that reduces the stray light entering the objective produces this oblique cone of illumination from 360 degrees. The oblique rays cross the specimen and are refracted and reflected by the specimen, producing a black background with objects brightly lit in the foreground (Chambers, Fellers, & Davidson, 2012) (Figure 1).

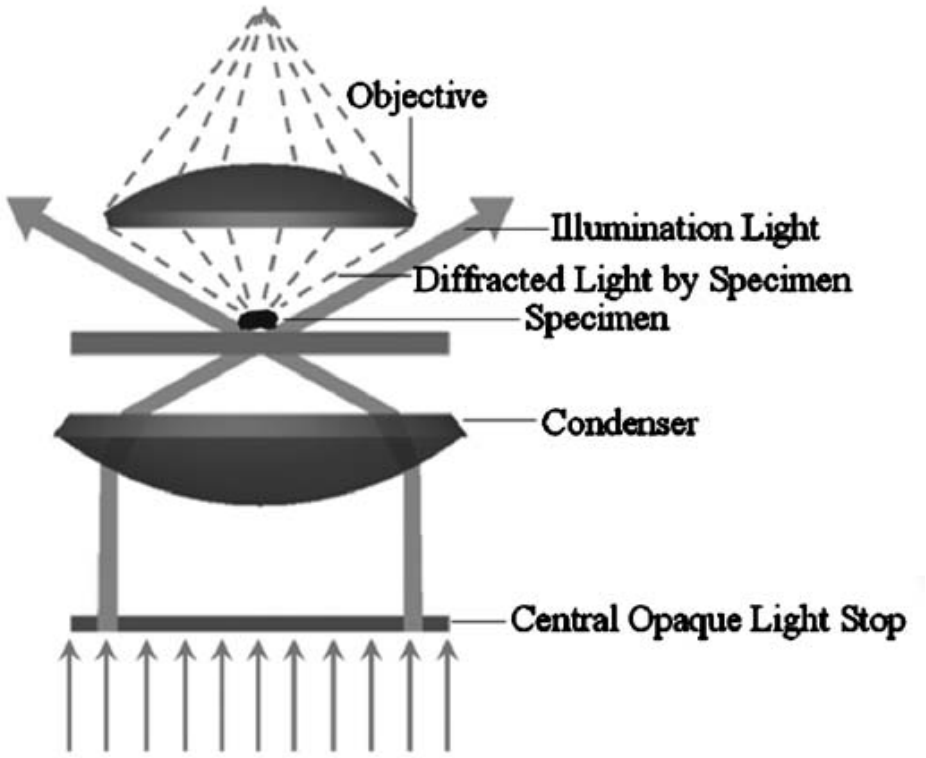


Figure 1. A schematic configuration of conventional darkfield microscopy. The darkfield condenser breaks illumination light so that light hits the specimen at oblique angles. Diffracted light from the specimen enters the objective lens giving the appearance of an illuminated specimen on a black background (Hu, Ma, & Liu, 2010).

Darkfield microscopy is useful in observing sub-microscopic particles (B. J. Ford, 2009) in transparent specimens such as minute organisms, fibres, hair, unstained bacteria, yeasts, protozoa and blood particles (Chambers et al., 2012).

Care needs to be taken in slide preparation in DM because features that lie above and below the plane of focus, such as fingerprints, dust, fibres or cleaning residue can scatter light and contribute to image degradation (Chambers et al., 2012).

2.1.1 Darkfield microscopy in dentistry.

Scientists and clinicians have been observing bacteria, human blood and body fluids with DM since the early 1900's (Elkes, Frazer, & Stewart, 1939). Clinically, DM has been used since the 1970's in dentistry (Macnab, 1976; Newman, 1985; Tomassini, Biscaro, & Frezzato, 1986). Other methods of evaluation of dental plaque samples are problematic, as the bacteria are difficult to distinguish from the diluent,

but DM enables the bacteria to be clearly identified and the patient treatment adjusted accordingly (Callens, 1992; Carrassi, Soragna, Onofri, & Abati, 1986; Chandrasekaran, Krishnaveni, & Chandrasekaran, 1998; Ullmann, 2001).

Some dentists use DM as a tool to inform patients about dental plaque with the aim of improving oral hygiene habits. Research into the success of the technique used in this way was inconclusive, despite many practitioners anecdotally citing it as a useful motivational tool (Drisko, White, Killoy, & Mayberry, 1987).

In 1988 Srivastava et al. carried out a study investigating sub-gingival plaque from healthy and diseased patients using DM and their results indicated ease of detection of pathogenic bacteria (Srivastava, Walsh, Basu, Glenwright, & Rippin, 1988). In the same year another study found DM to be of significant clinical value in the differential diagnosis of periodontal and endodontic abscesses (Trope, Tronstad, Rosenberg, & Listgarten, 1988).

2.1.2 Darkfield microscopy in microbiology.

In 1906, Landsteiner and Mucha were the first to recommend the use of darkfield microscopes to identify the presence of *Treponema pallidum*, which is responsible for causing syphilis, in serum exudate (Crissey & Denenholz, 1984; Pierce & Katz, 2011). Within a year, the DM technique became the routine investigation for syphilis diagnosis in Europe (Crissey & Denenholz, 1984; S., 1970). It is still utilised today and there are many current studies that still highlight the usefulness of DM in syphilis diagnosis (Cummings et al., 1996; Pierce & Katz, 2011; Wheeler, Agarwal, & Goh, 2004). In a 2004 retrospective study of 86 patients with primary (n=50) and secondary (n=36) syphilis, the authors concluded that DM investigation of serum extracted from a suspected syphilitic lesion was more diagnostically sensitive than serum treponemal enzyme immune assay (EIA) (Wheeler et al., 2004). Fast effective diagnosis allows treatment to be initiated immediately and can reduce the risk of the spread of infection.

The ease of detection of parasite-infected cells as seen through DM allows for easy malaria diagnosis (Jamjoom, 1991; Wood et al., 2009). New diagnostic

techniques have been developed such as saliva and urine antigen tests but in an earlier paper Jamjoom believed DM offered the “distinct advantages of rapid diagnosis, increased sensitivity, and adaptability to field work” (Jamjoom, 1983).

Darkfield microscopy is recommended as an adjunct to the IgM ELISA test for leptospirosis diagnosis (Sharma & Kalawat, 2008) and DM examination of diarrheal stool specimens is useful in the diagnosis of *Campylobacter enteritis* (Benenson, Islam, & Greenough III, 1964; Paisley, Mirrett, Lauer, Roe, & Reller, 1982). Darkfield microscopy is also being successfully used to monitor commercial yeast cultures and Wei et al (Wei, You, Friehs, Flaschel, & Nattkemper, 2007) state “the contrast of the images is higher than those taken by a light field in situ bright field microscope” allowing for more accurate monitoring.

2.2 Fresh Capillary Blood Analysis

Florence Sabin, in 1923, made observations of living human cells unstained and stained with supravital dyes. She reported that the “technique of studying living blood cells is so simple, that it is readily applicable to the study of clinical cases” (Sabin, 1923). In 1936 the haematologist Hansen-Pruss highlighted the usefulness of DM in viewing unstained blood samples (Hansen-Pruss, 1936). With the introduction of darkfield microscopes, early researchers gained a new insight into the anatomy and physiology of blood, such as revealing the first clear image of chylomicron remnants. Described by Edmunds in 1877 as 'blood-dust' or 'haemokonien', they were later discovered to be fat-laden particles from the digestion of food (Elkes et al., 1939). Early microscopic studies of blood coagulation were made using DM, as fibrin strands as well as the clustering of platelets can be distinctly imaged through this technique (Barratt, 1920). Todd and Barnetson (1988) highlight the use of darkfield microscopy in blood pathology research observing a number of organelles, granules and precipitates in red blood cells.

2.2.1 Günther Enderlein.

In 1925, Zoologist Günther Enderlein, published a comprehensive work titled *Bakterien-Cyklogenie* based on his research with FCB-DM, outlining his theory on microbial life cycles. Using FCB-DM he observed phenomena that could not be seen

in stained blood samples, and from observations he hypothesised that under conditions of environmental stress, many microbes and cells changed form in consistent ways. DNA was yet to be discovered in the 1920's so to explain his observations, Enderlein followed Antoine Bechamp's theory of pleomorphism (Bleker, 1993, p. 16; Teut et al., 2006). At the time Bechamp's work was controversial, and eventually the scientific community accepted the monomorphism concept of Louis Pasteur over pleomorphism.

Enderlein believed that some of the structures in the blood he observed were microbes that caused particular illnesses. He called this group of microbes *endobionts* (Bleker, 1993, p. 20). The smallest particles were called *protits*, which he believed to be small colloids of proteins, then in increasing size were *symprotits* and *macrosymprotits* (Teut et al., 2006).

Enderlein thought that these small particles were necessary for health homeostasis. His theories postulated that under pathological conditions such as intoxication or high acid load, these particles would polymerize into more complex units that he could observe in the blood. He believed every microorganism would undergo a development-cycle, that he called *cyclode* (bacterial cyclode) and that specific diseases were related to particular cyclodes (Ullmann, 2001). He's work mainly focussed on two cyclodes: the cyclode leading to the fungus *Mucor racemosus* that he linked to diseases concerning the blood, spine and rheumatism, and the cyclode leading to the fungus *Aspergillus niger* and diseases of lung, tuberculosis and cancer (Coyle, 2001).

His methods involved observation of dried, stained and live blood samples, which were often left for some hours before analysis. Many of the compounds he observed in the blood are today recognised as blood particle deterioration due to the drying process, or artefacts for example, non-blood particles such as dust, or compounds caused by unclean slides or poor sample preparation (McLaughlin et al., 2002). In the late 1990's biochemist Dr Christopher Gerner conducted research into the Enderlein technique at the University of Vienna. He identified the cellular forms observed by Enderlein as being primarily composed of cellular debris from degenerating red blood cells and that they were in fact globulin and albumin

molecules (Teut et al., 2006). Enderlein's theory, though thought valid at the time, does not stand up to modern scientific scrutiny (El-Safadi, Tinneberg, von Georgi, Munstedt, & Bruck, 2005; Ullmann, 2001). In fact, much of the criticism of FCB-DM stems from the work of Enderlein (Patterson, 2012).

There is a scarcity of research, not only on live blood analysis, but specifically Enderlein's technique. A 2006 study aimed at testing the reliability of Enderlein's blood analysis included only two experienced practitioners (Teut et al., 2006). They concluded there was limited accuracy and inter-observer reliability in the technique, affected potentially by the small scale of the study, and raised a number of valid criticisms of the Enderlein technique. These included the uneven distribution of blood over the slide that would affect the drying time of the blood and change the diagnostic conclusions; slide preparation and cleaning that left residue that may be read as diagnostically significant, and blood reactivity to glass over time, causing fibrin development.

Another study in 2002 sought to investigate the efficacy of Enderlein's technique in observing and diagnosing various cancers in blood samples. Out of the 110 samples studied, 3 of the 12 patients with tumour metastases were detected, and the authors concluded the technique did not seem to reliably detect the presence of cancer (El-Safadi et al., 2005). This may well be the case, but both of these studies have used very small sample numbers deeming any significance unlikely. Neither of these studies made comparisons with conventional blood pathology.

2.2.2 Philip Hoekstra.

Philip Hoekstra developed the FCB-DM technique termed Hemaview^{TM} in the 1970's in the United States. He developed live blood analysis models more in line with orthodox pathology techniques to help alleviate technical issues, improve the validity of the technique, and so that information about the patient could be understood by all types of health practitioners (Metagenics, 2010).

Hoekstra, in a paper presented at the Live Cell Analysis System Symposium in Tokyo in 1987,compared two peripheral blood specimens from 25 patients – two

Wright's stained blood smears and two wet-mounts immediately analysed by a FCB-DM practitioner. He found that the linear regression analysis of the data from the two techniques demonstrated no significant difference between the wet-mount and the blood smear for acanthocytes, echinocytes, elliptocytes, macrocytes, and neutrophilic hypersegmentation (Hoekstra, 1987).

The key differences between Hokestra's Hemaview^{TM} technique analysed in this study and Enderlein's work lie not only in the interpretation of findings but in the duration of the observational window. In the Hemaview^{TM} technique the capillary blood sample is analysed within 15 minutes of sample collection.

2.2.3 Current literature and research.

There has been very little research published regarding FCB-DM and its use as a clinical screening tool and thus, there is very little information about its validity. There are a small number of unpublished papers reporting on research from Australia, New Zealand and Japan.

One large study in 1960 compared a wet capillary blood preparation observed under DM with 'standard blood chemistry' in Japanese men and women. The aim of the study was to investigate the clinical usefulness of FCB-DM in health care and evaluate "its sensitivity in discerning aberrant lipid and protein metabolism, detection of anaemia, and assessment of general level of health" (Oshima, Tsuruoka, Maeda, Hamada, & Takagi, 1987). This research utilised a grading system for FCB-DM analysis where numbers from 0 to 4 related to the extent a variable was observed, and then this data was converted to a health rating scale – 'healthy', 'semi-healthy' and 'unhealthy'. The authors demonstrated "equal sensitivity between darkfield microscopy and standard chemistry analysis for the variables studied". These variables included observations of white cell populations, liver function, cholesterol, blood sugar levels and red cell variables. However, the authors did not provide a clear and detailed description on how these variables were measured and what exact comparisons and correlations were made in the study, therefore the validity of their grading system is questionable.

A more recent study looking into FCB-DM accuracy using the Hemaview™ method found positive correlations between 13 different practitioners identifying an extensive range of variables (Petric, Aylett, & Hope, 2010). This study aimed to demonstrate practitioner's congruence for each variable but was limited by the lack of a test control or comparison to other testing methods such as acknowledged standard venous blood pathology. Thus, this study demonstrated inter-practitioner reliability rather than accuracy of the technique.

A number of conference papers and small-scale studies have been carried out in recent years in Australia. These have demonstrated the use of FCB-DM in observing nutrient deficiencies, inflammation, erythrocyte oxidation and abnormal white blood cells (Reeve & Reilly, 2011; Wayne Reilly, 2009; Reilly & Mannion, 2009, 2010; Reilly et al., 2010). All these studies observed positive relationships between pathology markers and FCB-DM but are mostly retrospective clinical observations, and report on single patients or small cohorts.

2.2.4 Point of care screening.

Larson (Carey et al., 1997) stated that a useful point of care screening test should fulfill the following criteria: that it provide early detection of disease, that the disease being screened for poses a significant health problem, that the disease being screened for can be treated and that it be simple, cost effective, reliable and convenient to use.

A 2005 rural community-based study monitoring diabetes in patients found that the point of care testing improved patient outcomes (Shephard et al., 2005). This study followed 54 participants with established diabetes whose finger prick capillary HbA1c, blood lipid and glucose were monitored at the point of care. Over the period of 12 months these patients had a 30% greater increase in glycaemic control than patients who were not monitored at point of care. A patient satisfaction survey of the point of care screening in general practice reported increased motivation for management of chronic conditions (Laurence et al., 2008).

However, studies of the use of DM in the clinical dental setting (Cole,

Newcomb, & Nixon, 1984) found no alteration to patient motivation and treatment compliance with patients exposed to DM observations of their plaque and salivary bacteria. This was a study on a small sample of 14 patients, and the researchers recognised the benefit of using DM in patient education, as a number of the participants' oral hygiene improved on observing dental plaque.

According to Triveri (2002) the observation of the shape of red blood cells, white blood cells and platelets enables the FCB-DM practitioner to detect early signs of disease. If this is the case, then the clinical application of FCB-DM lends itself to use in monitoring health conditions such as metabolic syndrome.

2.3 Pathology Techniques

It is recognised that the automated pathology systems are faster and more cost effective for the counting of white blood cells, red blood cells and platelets, whereas visual microscopy is superior for differentiating cells based on nuances of cellular features, and especially for recognising immature cells (Barnes, McFadden, Machin, & Simson, 2005). Manual counting and observation of blood components has the advantage in that it can extrapolate quantitative and qualitative assessment of blood cells. Blood films, which can be manually or mechanically analysed, are assessed by similar techniques to FCB-DM. The main point of difference between blood films and FCB-DM is that blood films are prepared using blood treated with anticoagulants and stains to aid analysis, whereas FCB-DM observes fresh blood and has greater resolution, contrast and acuity than analysis using light-field microscopes (Chambers et al., 2012).

2.3.1 Blood films.

Blood film analysis has remained as one of the world's most widely and frequently used pathology tests and has undergone few changes since its evolution in the 1800's (Houwen, 2002; S. Jung et al., 2010). With the development of more sophisticated automated blood-cell analysers, the proportion of blood films has steadily diminished (Gutierrez, Merino, Domingo, Jou, & Reverter, 2008), nevertheless, the blood smear remains a crucial diagnostic aid for certain infections and in the differential diagnosis of anaemia and thrombocytopenia and in the

identification and characterisation of leukaemia and lymphoma (Bain, 2005)(Table 1).

Table 1. Clinical indications for blood smear analysis request.
(Bain, 2005)

• Features suggestive of anaemia, unexplained jaundice, or both
• Features suggestive of sickle cell disease — dactylitis or sudden splenic enlargement and pallor in a young child or, in an older child or adult, limb, abdominal, or chest pain
• Features suggestive of thrombocytopenia (e.g., petechiae or abnormal bruising) or neutropenia (e.g., unexpected or severe infection)
• Features suggestive of a lymphoma or other lymphoproliferative disorder — lymphadenopathy, splenomegaly, enlargement of the thymus (a mediastinal mass on radiology) or other lymphoid organs, skin lesions suggestive of infiltration, bone pain, and systemic symptoms such as fever, sweating, itching, and weight loss
• Features suggestive of a myeloproliferative disease — splenomegaly, plethora, itching, or weight loss
• Suspicion of disseminated intravascular coagulation
• Acute or recent-onset renal failure or unexplained renal enlargement, particularly in a child
• On retinal examination, haemorrhages, exudates, signs of hyper viscosity, or optic atrophy
• Suspicion of a bacterial or parasitic disease that can be diagnosed from a blood smear
• Features suggestive of disseminated nonhaematopoietic cancer — weight loss, malaise, bone pain
• General ill health, often with malaise and fever, suggesting infectious mononucleosis or other viral infection or inflammatory or malignant disease

There are some issues with blood films that relate mainly to their preparation. Certain cell types can be damaged easily, and the wedge methods, commonly utilised, can cause an uneven distribution of different cell types, especially monocytes and other large leukocytes, that are, during the preparation process, pushed to one end of the spread of blood over the slide (Houwen, 2002). This leads to a 5% to 10% underestimation of monocyte presence when compared with monoclonal antibody-based flow cytometry differential counts (Houwen, 2002). The blood sample is left to dry before being fixed onto the slide by immersion in methanol. The fixed film of blood is then stained with a mixture of several dyes to enable identification of individual cells (Bain, 2005).

When any leukocyte suspension is subject to drying on the slide the cells undergo shrinkage and pyknosis where condensation of chromatin in the nucleus causes the cell to undergo necrosis (Cuadra, 1978). As the sample dries the fluid becomes increasingly hypertonic causing further shrinkage and necrosis (Cuadra, 1978).

Leukocytes, while floating in physiological fluid, are spherical in shape, but when released from their suspending fluid, they spread flat because the capillary force between the cell membrane and the glass overcomes the surface tension of the cell (Cuadra, 1978). This spreading allows the morphology of the cell to be observed readily, a major benefit of blood film analysis.

A study in 1985 evaluated the manual differential white cell count performed by 73 technicians and found correlations with automated blood pathology counts for neutrophils, lymphocytes and eosinophils (Koepke, Dotson, & Shifman, 1985). Variability was noted for atypical lymphocytes and monocytes. The sensitivity of the manual differential white cell count for clinically important conditions ranged from 100% to 34%.

Another study comparing a 100 cell manual differential white blood cell count and an automated differential white cell count of blood films in 136 samples, reported similar precision between the two counting methods. The correlation values between the manual and automated counts were $r^2 > .9$ for neutrophils and lymphocytes, monocytes $r^2 = .81$ and eosinophils $r^2 = .67$, indicating strong correlations (Briggs et al., 2007).

2.3.2 Automated blood analysers.

The French anatomist and histologist Louis-Charles Malassez developed a blood cell counting technique in the late 19th century upon which the modern automated cell counters are based (Verso, 1964). After a number of notable modifications in 1947, Swedish Professor of Medicine, Carl Magnus Lagercrantz, developed a sensitive and powerful photomultiplier tube utilising dark ground illumination, which enabled him to be the first to count erythrocytes with a machine

(Crosland-Taylor, Stewart, & Haggis, 1958; MacFarlane, Shillitoe, Watson-Williams, & Meynell, 1957).

Hans Baruch, an American physiologist, invented a “robot chemist” in 1959. This machine used “discrete sample analysis” allowing lower reagent consumption and shorter manual processing times (Rosenfeld, 1999).These early machines were slow with only partial automated capacity, offering only limited assistance to the manual practices (Briggs et al., 2007). Since then there have been many developments in the automated analyser and cell counter technology and improvements in efficacy and accuracy.

Today, there are a number of different types of clinical blood analysers used for quantitative buffy coat analysis, automated impedance analysis, digital image processing and flow cytometry analysis (DeNicola, 2011).

For Digital Image Processors, stained blood films are analysed by a computer driven automated microscope and leukocytes are classified and counted (Bentley, 1990). Early models were slow and there were difficulties in identifying specific cell types and abnormal cells, but developments in technology have produced analysers with precision similar to that of the 100 cell manual count (Briggs et al., 2007).

In the 1950’s, American biochemist Leonard Skegges developed the Continuous Flow Analysis (CFA), or flow cytochemistry machine. This machine utilises air bubbles, anticoagulants and reagents to separate a continuously flowing stream of blood (Rosenfeld, 1999). CFA machines are the most commonly used machines in pathology labs today and are considered the acknowledged standard in automated haematology (DeNicola, 2011). These analysers use a combination of darkfield and bright field light scattering that focus on the counting sensor (Saunders, 1972). These machines measure the components of a full blood count including liver function, electrolytes, glucose, serum albumin, creatinine, and iron levels.

In 1974, the chemists Jarda Ruzicka and Elo Harald Hansen developed a related technique termed flow injection analysis (FIA) (Ruzicka & Hansen, 2008) where a plug of sample is inserted into a flowing carrier stream. The principle is similar to CFA but no air is injected into the sample or reagent streams. In CFA each

segment of sample is equally proportioned with reagent, and mixing within each segment is improved by the microcirculation pattern of the flowing stream (Patton & Crouch, 1986) whereas in FIA, mixing of the sample with reagent can take longer. These differences are significant when dealing with samples that require mixing with reagents to analyse the resulting reaction (Patton & Crouch, 1986; Snyder, 1980), but have little impact on haematological analysis.

Automated haematology machines that can analyse finger prick capillary blood exist, and with ease of sample extraction (compared to venous blood sample extraction) have the potential to facilitate patient management. In a 2012 study (Ponampalam, Fook-Chong, & Tan, 2012), 314 emergency department patients' capillary and venous blood samples were analysed and compared. The authors found strong correlations between the white blood cells, haemoglobin and platelet counts, as measured by the automated venous blood analysis and the capillary blood machine analysis (Osei-Bimpong, Jury, McLean, & Lewis, 2009).

Other capillary testing techniques have been developed as viable alternatives to venous analysis. For example, the Demecal set (Demecal Europe, Haarlem, The Netherlands) that allows medically untrained people to take a finger prick sample and send it off to be analysed (Gootjes, Tel, Bergkamp, & Gorgels, 2009) or the HemoCue white blood cell analyser that has shown "good precision for replicate measurements on blood samples over a range of white blood cell counts" (Osei-Bimpong et al., 2009).

2.4 Capillary and Venous Blood Compared

Determining blood cell counts from venous blood samples is acknowledged as the gold standard in pathology and considered the most accurate representation of blood constituents (Yang et al., 2001). Capillary blood collections have developed as an alternative to venepuncture notably in paediatrics and in field medicine since it requires only a small amount of blood for analysis (Schalk, Scheinpflug, & Mohren, 2009). Only a small number of laboratories have undertaken analysis of capillary blood. Differences between capillary and venous blood samples are likely to exist, but the extent of the differences and the impact on clinical application is questionable.

There is conflicting information in the literature as to differences in blood cell constituents between venous and capillary blood samples. Numerous studies have shown that the total leukocyte counts are higher in capillary blood than venous samples (L. N. Daae, Halvorsen, Mathisen, & Mironska, 1988; Jouault et al., 1990; Schalk, Heim, Koenigsmann, & Jentsch Ullrich, 2007; Schalk, Scheinpflug, & Mohren, 2008; Yang et al., 2001). Studies of full blood counts in healthy adults using automated haematological analysers have found mean leukocyte counts are 7.8% (L. N. Daae et al., 1988) and 8.34% (Yang et al., 2001) higher in finger prick blood than venous samples. Variations were the greatest among the large leukocyte population (principally neutrophils) and lowest among small leukocytes (principally lymphocytes) (C. A. MacLennan et al., 2007).

This variation was also observed in a number of studies investigating finger prick capillary blood sampling for monitoring of CD4 numbers in HIV patients (Cracknell, Hinchliffe, & Lilleyman, 1995; C. A. MacLennan et al., 2007). Despite the variances the authors concluded that finger prick blood analysis is effective and accurate, and less invasive than venous blood samples.

In contrast, Dreyer et al. (Dreyer, Pillay, & Jacobs, 1994) analysed capillary and venous samples from 50 adults and found no difference in mean total white cell counts and the differential spread was concordant between the two samples. In two other studies (Schalk et al., 2008; H. B. Tsai et al., 2012), pairs of capillary and venous blood samples from haematologic and healthy patients were analysed using automated flow cytometry analysers. No statistically significant differences were seen in the white cell and absolute neutrophil counts in either of the groups.

Another study comparing results of 310 patients' finger prick capillary and venous blood samples found no significant differences between the mean levels of all complete blood count variables analysed, including white cell differential counts and haemoglobin (Rao, Moiles, & Snyder, 2011).

Schalk et al. (2007) investigated 463 capillary and venous blood samples from haematologic and healthy patients. They investigated haemoglobin, haematocrit, white blood cells, platelets, red blood cells, mean corpuscular volume, mean

corpuscular haemoglobin, and mean corpuscular haemoglobin concentration using a haematology analyser. A higher value in capillary white cell counts (+0.2 X 10^9/L) compared to venous samples was observed. However, they found the correlation between the capillary and venous values was very high for most of the eight variables investigated (on average $r = 0.95$).

In 2009, Schalk et al. published a review of the literature investigating capillary and venous blood count variables (2009). The study populations included children and adults, healthy and sick. They found that on average, the capillary white cell values were above the corresponding venous values by 9.5% (1.2 x 10^3/ μL). These differences were higher in children at 14.6 % (2.0 x 10^3/ μL) than in adults at 2.7% (0.2 x 10^3/ μL). Capillary absolute neutrophil counts were on average 8.9% (0.68 x 10^3/ μL) higher than the corresponding venous count, again higher in children (11%, 0.91 x 10^3/ μL) than in adults (4.7%, 0.22 x 10^3/ μL) (Schalk et al., 2009).

One explanation of this variation is the rapid laminar flow through the blood vessels, the central section having the highest velocity (L. N. Daae et al., 1988; N. W. Daae, 1989; Schalk et al., 2009; Vejlens, 1938). Erythrocytes and leukocytes are normally concentrated in this central and more rapid stream while platelets and plasma move more slowly along the vessel wall (N. W. Daae, 1989; Vejlens, 1938). Thus, whereas erythrocytes and leukocytes are likely to be concentrated in the skin puncture blood, this phenomenon may well be levelled out with increased volume of blood in venous samples. This phenomenon is more pronounced with exposure to the cold, most likely with bleeding from the contracted vessel accounting for the higher white cell numbers in an ear lobe prick (L. N. Daae et al., 1988; L. N. W. Daae, Hallerud, & Halvorsen, 1991; Schalk et al., 2009). Another explanation might be related to haemo-concentration caused by capillary fluid transudation (L. N. W. Daae et al., 1991; Kayiran, Özbek, Turan, & Gürakan, 2003).

Capillary blood sampling is perceived by the patient as less traumatic or painful and more convenient and faster than venous sampling (Woods et al., 2004). The process does not require active participation of the patient such as making a fist or extending the arm and post procedural infection or complications are less likely (Schalk et al., 2009).

2.5 Determining the Validity of FCB-DM

The aim of the study was to determine the validity of FCB-DM by examining the relationship between FCB-DM variables and venous blood pathology tests when screening for metabolic syndrome risk factors. Validity, in science, is the extent to which a concept or measurement corresponds accurately to the real world (Brians, Manheim, Willnat, & Rich, 2010, p. 125). Büttner (1997) states the validity of laboratory testing is the 'degree of achieving the objective', a test that answers the question the physician in clinical practice asks.

There are a number of different types of validity: Content, Construct and Criterion (Salkind, 2011, p. 36). Content validity, usually used in the field of education research (George et al., 2003), is a measure of the extent to which a test accurately reflects all possible topics within one idea or subject. Construct validity examines how well a test reflects an underlying construct or idea (Salkind, 2011, p. 37). Criterion validity includes both predictive and concurrent validity and 'reflects the use of a criterion to create a new measurement procedure' (Lund & Lund, 2013). Predictive validity measures how well a test predicts a criterion, while, Concurrent validity measures how well a particular test correlates with a previously validated measure (George et al., 2003; Hurley et al., 2011; Salkind, 2012) where the measurements are carried out at the same time.

Concurrent validity, in the context of this study, refers to the strength of association, or correlation, between FCB-DM measures of specific variables in comparison to the relevant acknowledged standard venous blood pathology measures (Karras, 1997). To test concurrent validity, data analysis needs to examine the relationship between FCB-DM and venous blood pathology through correlation coefficients (Field, 2009, p. 167; Salkind, 2012, p. 125). The correlation coefficient (r) can only take on values of -1 to +1 indicating a positive or negative correlation. The value indicates the strength of the relationship, where 1 represents a perfect correlation and a correlation of 0 indicates no relationship between the variables (Pallant, 2007, p. 133). A significant correlation assumes the relationship between variables is meaningful, but in reality may lack clinical relevance, thus a degree of discernment in interpretation is required (Salkind, 2012, p. 125).

2.6 The Global Epidemic of Metabolic Syndrome and Obesity

Obesity is a chronic disease affecting all age groups and all regions of the world. Even in the early 20th century, obesity was recognised to increase death rate in studies of life insurance (Cordero et al., 2006). Obesity, now considered a chronic disease, has been shown to increase the risk of general mortality and morbidity (Binns & Low, 2013; Danaei et al., 2011; Finucane et al., 2011; Withrow & Alter, 2011; World Health Organisation, 2000b). The International Diabetes Federation has reported obesity to be one of the main drivers of the increasing incidence of metabolic syndrome (K. G. M. M. Alberti et al., 2006).

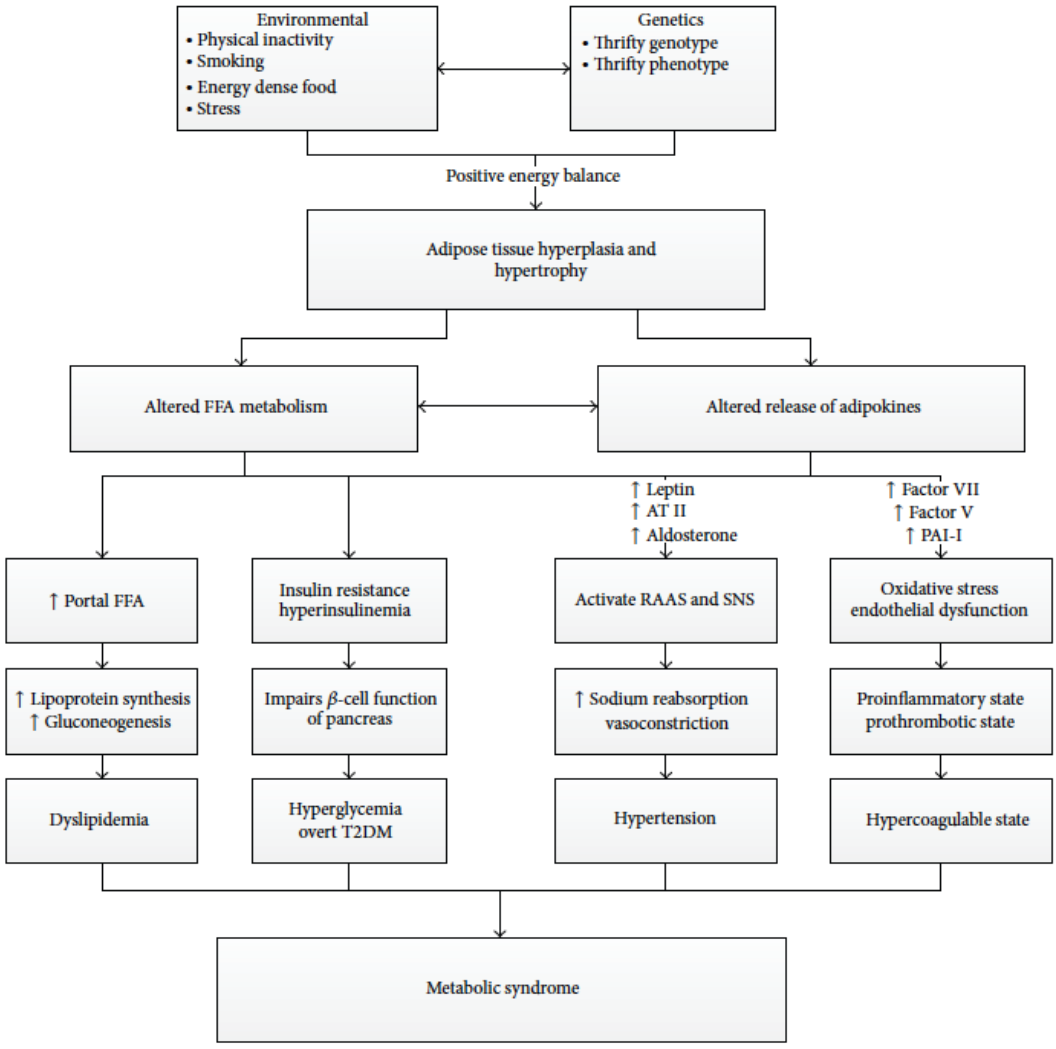


Figure 2. A schematic presentation of the progression and development of metabolic syndrome. (Kaur, 2014)

Metabolic syndrome is a collection of interconnected physiological, biochemical, clinical and metabolic factors that increase the risk of diabetes, cardiovascular disease, chronic inflammatory conditions and all causes of mortality (K. G. M. M. Alberti et al., 2006; Dominiczak, 2003a; S. M. Grundy et al., 2004; Kaur, 2014; Samson & Garber, 2014; Shoelson et al., 2007).

Obesity, especially visceral adiposity, is directly related to the pathogenesis of metabolic syndrome, triggering factors that cause changes to normal homeostasis (Aballay, Eynard, Díaz, Navarro, & Muñoz, 2013) (Figure 2). These include high plasma levels of pro-inflammatory cytokines and fatty acids, which together appear to be responsible for the development of metabolic syndrome and insulin resistance (Aballay et al., 2013; Ros Pérez & Medina-Gómez, 2011). It is well understood that patients with high waist measurement, high waist to hip ratio and/or high body mass index (BMI) are at significantly increased risk of metabolic syndrome.

Due to the complexity of the clinical picture of metabolic syndrome, there are no internationally agreed diagnosis criteria (Alshehri, 2010; S. M. Grundy et al., 2005; Kaur, 2014; Ritchie & Connell, 2007). The WHO, the American Heart Foundation (AHF) and the International Diabetes Foundation (IDF) provide the three diagnostic criteria most prevalent in the literature (Table 2). The WHO criteria emphasise insulin resistance as the major underlying risk factor, though this can be difficult to measure directly in a clinical setting (S. M. Grundy et al., 2005).

A joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity, published in 2009, aimed to address the issues with the WHO diagnostic criteria of metabolic syndrome (K. G. Alberti et al., 2009). They agreed that waist measurement would continue to be a useful preliminary screening tool, though internationally recognised waist circumference thresholds for abdominal obesity should be applied (Appendix E).

Table 2. Criteria for clinical diagnosis of metabolic syndrome.
(S. M. Grundy, 2005; S. M. Grundy et al., 2005; Kaur, 2014; Ritchie & Connell, 2007; Samson & Garber, 2014)

Clinical measures	WHO (1998)	AHA (2005)	IDF (2005) and Joint Task Force (2009)
Insulin resistance	IGT, IFG, type 2 Diabetes or Lowered insulin sensitivity	n/a	n/a
	Plus any two of the following	**Any two of the following**	
Waist circumference	> 0.85 waist to hip ratio in women > 0.90 waist to hip ratio in men and/ or BMI > 30 kg/m^2	≥88 cm (≥35 inches) in women ≥102 cm (≥40 inches) in men	Increased waist circumference (population specific) **Plus any two of the following**
Triglycerides	≥150 mg/dL (1.7 mmol/L)	≥150 mg/dL (1.7 mmol/L) or On drug treatment for elevated triglycerides	≥150 mg/dL (1.7 mmol/L) or On drug treatment for elevated triglycerides
HDL	<39 mg/dL (1.3 mmol/L) in women <35 mg/dL (1.03 mmol/L) in men	<50 mg/dL (1.3 mmol/L) in women <40 mg/dL (1.03 mmol/L) in men or On drug treatment for reduced HDL	<50 mg/dL (1.3 mmol/L) in women <40 mg/dL (1.03 mmol/L) in men or On drug treatment for reduced HDL
Blood pressure	≥140/90 mm Hg	≥130 mm Hg systolic blood pressure or ≥85 mm Hg diastolic blood pressure or On antihypertensive drug treatment in a patient with a history of hypertension	≥130 mm Hg systolic blood pressure or ≥85 mm Hg diastolic blood pressure or On antihypertensive drug treatment
Glucose	IGT, IFG, type 2 Diabetes	≥100 mg/dL or On drug treatment for elevated glucose	≥100 mg/dL

*IGT – Impaired glucose tolerance *HDL – High density lipoprotein *IFG – Impaired fasting glucose *BMI – Body mass index

2.6.1 Epidemiology

Obesity and metabolic dysfunction are major global health issues today (Barquera et al., 2013; Binns & Low, 2013; Lal, Moodie, Ashton, Siahpush, & Swinburn, 2012; Swinburn, 2003; World Health Organisation, 2000a, 2000b, 2015). The prevalence of obesity has steadily increased over the last 30 years worldwide. In a recent review Kaur (2014) reports that the incidence of metabolic syndrome ranges from 10 - 84% worldwide, depending on region, population, ethnicity and gender.

In Australia, high BMI has overtaken smoking and high blood pressure to be the leading risk factor for the burden of disease (Ananthapavan, Sacks, Moodie, & Carter, 2014). Australia has the fifth highest rate of obesity in the world behind USA, Mexico, New Zealand and the United Kingdom (Australian Bureau of Statistics, 2011). The 2011 - 2012 Australian Health Survey reported 63.4% of Australians over the age of 18 had a BMI in the overweight or obese categories (Australian Bureau of Statistics, 2013). Over the last 20 years, Australia has seen a steady increase in the national BMI (Figure 3). The longitudinal population-based *Australian Diabetes, Obesity and Lifestyle Study*, surveyed 5985 men and women in 1999/2000 and again in 2004/2005 (Adrian J Cameron et al., 2003). They found an association between obesity and deterioration in mental and physical health. Health related quality of life was also found to be a predictor of weight gain over the five-year period, indicating a positive association between obesity and health deterioration (A. J. Cameron et al., 2012).

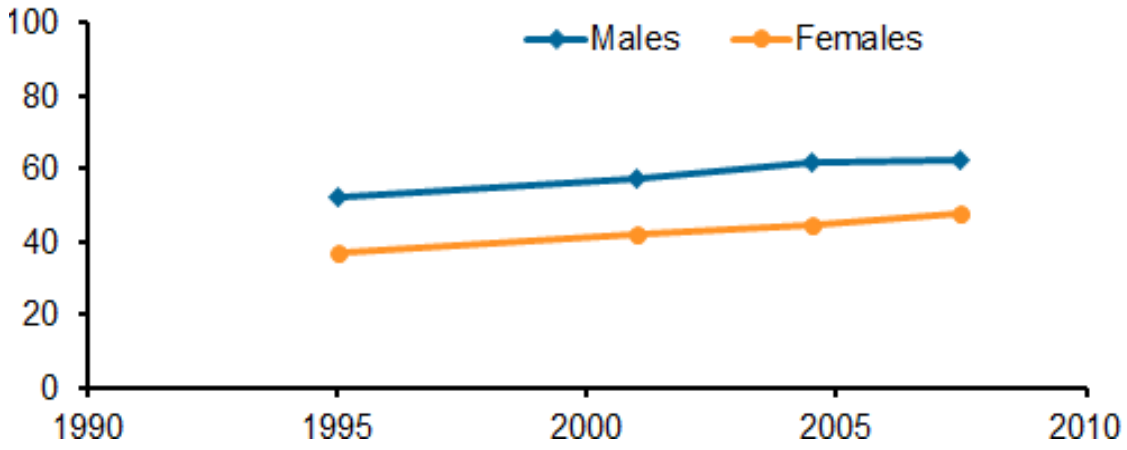


Figure 3. Increasing BMI in Australia.
Percentage of overweight and obese adults over a 10 year period (Australian Bureau of Statistics, 2007; Welfare., 2015).

2.6.2 The rising cost of obesity.

The *Global Burden of Metabolic Risk Factors of Chronic Diseases Collaborating Group (Blood Glucose)* published a number of reviews in The Lancet in 2011 (Danaei et al., 2011; Finucane et al., 2011). They examined plasma glucose results from 1980 - 2008 in 2.7 million people and demonstrated that hyperglycaemia and diabetes were a 'rising global hazard' and that the number of adults with diabetes more than doubled over the last 30 years (Danaei et al., 2011). They reported the age-standardised mean BMI in Australian men increased from 24.9 in 1980 to 26.7 in 2008, and from 23.6 to 26.9 in Australian women for the same period (Finucane et al., 2011). Following the World Health Organisation BMI guidelines (Table 3), these results place the average Australian in the overweight category.

Obesity manifests an increase in risk factors for a number of chronic illnesses placing a heavy burden on healthcare systems worldwide due to an increase in healthcare utilisation (Lehnert, Sonntag, Konnopka, Riedel-Heller, & König, 2013). In a review of the direct costs of worldwide obesity, obese patients were found to accumulate approximately 30% higher medical costs than those with normal weight (Withrow & Alter, 2011).

In 2005, the Medical Journal of Australia reported that the direct cost of the overweight and obese on the Australian health care system was $21 billion (Colagiuri et al., 2010) and the annual cost of health care per obese person was almost double ($2788) that of healthy weight person ($1472) (Binns & Low, 2013). The Australian Bureau of Statistics estimated the overall cost of obesity to Australian society and government in 2008 was $58.2 billion (Australian Bureau of Statistics, 2011).

2.6.3 Obesity, metabolic syndrome and insulin resistance.

Obesity is associated with an increased risk of developing insulin resistance, hypertension, lipid abnormalities, atherosclerosis and diabetes (Eckel et al., 2005). All of these risk factors are included in the diagnostic criteria of metabolic syndrome (Table 2). Compared to subcutaneous fat, visceral adipose tissue has specific gene expression patterns that are associated with insulin resistance (Samson & Garber, 2014). Visceral adipose tissue produces metabolically active substances that elicit local and systemic effects on the body (Bastard et al., 2006), for instance, raised levels

and increased turnover of non-esterified fatty acids. These free fatty acids affect insulin signalling pathways in the liver, skeletal muscles and blood vessels that in turn lead to hyperglycaemia and endothelial dysfunction (Johnson & Weinstock, 2006; Reaven, 1988). These changes accelerate gluconeogenesis, decrease glucose uptake in skeletal cells, increase platelet aggregation and increase oxidative stress and the formation of advanced glycation end products (Johnson & Weinstock, 2006; Keller, 2006).

There are other diseases that have been reported to be associated with insulin resistance including polycystic ovarian syndrome, non-alcoholic fatty liver disease, hypertension, sleep apnoea and certain forms of cancer (Johnson & Weinstock, 2006). Although visceral adiposity is a risk factor for insulin resistance, not all overweight patients have insulin resistance (Despres, 2006). Despres and Lemieux pose the question – is visceral adiposity 'a causal factor or simply a marker of a dysmetabolic profile?' (Despres & Lemieux, 2006).

2.6.4 Obesity, metabolic syndrome and inflammation.

It is understood that low grade inflammation and immune system activation are involved in the pathogenesis of obesity-related metabolic changes such as insulin resistance (Emanuela et al., 2012; Esser, Legrand-Poels, Piette, Scheen, & Paquot, 2014). Proinflammatory cytokines have been shown to be over-expressed in obesity (Emanuela et al., 2012; Hotamisligil, 2006). This increase in cytokines in the extracellular environment induces overall systemic inflammation (Emanuela et al., 2012). The presence of obesity causes an infiltration of macrophages and other immune cells into adipose, pancreatic, liver and muscle cells leading to systemic inflammation (Esser et al., 2014).

The high vascularity of the liver means that adipocytes are in close proximity to immune cells such as Kupffer cells and macrophages. Hotamisligil (2006) states that this structure enables a dynamic interaction between immune and metabolic responses that may contribute to the initiation and development of insulin resistance and metabolic disease. Increased plasma CRP, fibrinogen and total white blood cell counts were observed in patients with metabolic syndrome compared to healthy

controls (E. S. Ford, 2003). It has been suggested that the increased levels of proinflammatory cytokines produced by adipose tissue, such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α, play an important role in the development of insulin resistance (Bastard et al., 2006; Kahn, Hull, & Utzschneider, 2006; Wellen & Hotamisligil, 2005).

Xu et al. (2003) found that many proinflammatory and macrophage-specific genes are significantly up regulated in white adipose tissue in mouse models of genetic and high-fat diet-induced obesity. The macrophages in adipose tissue are bone marrow-derived and their number is strongly correlated with bodyweight, body mass index and total body fat (Subramanian & Ferrante, 2009). This macrophage-related proinflammatory activation may contribute to the pathogenesis of obesity-induced insulin resistance (Balistreri, Caruso, & Candore, 2010; de Heredia et al., 2012; Subramanian & Ferrante, 2009; Xu et al., 2003).

Obesity is also known to impair immune function through altering leukocyte counts as well as cell-mediated immune responses. In addition, evidence has arisen that altered immune function contributes to the pathogenesis of obesity (de Heredia et al., 2012; E. S. Ford, 2003).

2.6.5 Obesity, metabolic syndrome and hyperlipidaemia.

Insulin resistance and dyslipidaemia are established risk factors for metabolic syndrome (Dominiczak, 2003b; Eckel et al., 2005; Pannier et al., 2006; Samson & Garber, 2014). Lipoprotein abnormalities associated with insulin resistance include hypertriglyceridaemia and high levels of very low density lipoprotein (VLDL) with low levels of high density lipoprotein (HDL) and apolipoprotein A (Garg, 1996). The higher concentrations of oxidized low density lipoprotein (LDL) and triglycerides have been associated with an increased incidence of metabolic syndrome, as well as its components of abdominal obesity and hyperglycaemia (Holvoet, Lee, Steffes, Gross, & Jacobs, 2008).

Lipoprotein lipase (LPL) hydrolyses triglycerides and is involved in promoting the cellular uptake of chylomicron remnants, cholesterol-rich lipoproteins, and free fatty acids in tissue (Eckel, Prasad, Kern, & Marshall, 1984; Mead, Irvine, &

Ramji, 2002; Rinninger et al., 1998). In healthy individuals insulin has a stimulating effect on the activity of LPL but in overweight individuals with insulin resistance, adipose tissue LPL responsiveness is reduced. This lowered LPL activity in insulin resistant states may reduce VLDL catabolism and accentuate hypertriglyceridaemia (Eckel & Yost, 1987; Garg, 1996). It is also postulated that a reduction in insulin responses may result in excessive tissue lipolysis and an increase in free fatty acids in the liver, driving up triglyceride synthesis (Avramoglu, Qiu, & Adeli, 2003; Zammit, 1996).

Some studies suggest that high concentrations of triglyceride rich VLDL particles may impair insulin action by inhibiting binding to the insulin receptor (Berliner, Frank, Karasic, & Capdeville, 1984; Karpe, Dickmann, & Frayn, 2011; Samuel & Shulman, 2012; Sparks, Sparks, & Adeli, 2012; Steiner & Vranic, 1982) indicating that primary dyslipidaemia leads to insulin resistance. Alternatively, Garg et al. (1988) showed that patients with hypertriglyceridaemia were likely to be insulin resistant, and that lowering plasma triglyceride levels in these patients did not improve the insulin-mediated glucose disposal, indicating that insulin resistance is the underlying mechanism. In more recent reviews the complexity and interdependent involvement of multiple endocrine and inflammatory pathways is acknowledged in metabolic syndrome (Glass & Olefsky, 2012; Qatanani & Lazar, 2007).

Risk factors for atherosclerosis such as dyslipidaemia and changes in insulin and glucose metabolism have been extensively reported in patients with abdominal adiposity (Abbott et al., 1987; Berliner et al., 1984; Cassader et al., 2001; Chahil & Ginsberg, 2006; Frayn, 1993; Garg, 1996; Karhapää, Malkki, & Laakso, 1994; Laakso, Sarlund, & Mykkänen, 1990; Pouliot et al., 1994; Steiner & Vranic, 1982).

2.6.6 Anthropometric measurements.

It is understood that an individual's weight is a fairly meaningless indicator of adiposity, unless it is related to the individual's height, race, build or age (World Health Organisation, 1995), and it is actually the distribution of fat in the body that affects the health risks associated with obesity (World Health Organisation, 2000a). Anthropometric calculations often combine measures, for instance in the body mass

index (BMI). This calculation of weight-to-height ratio, weight (kg)/ height^2 (m), is used clinically to classify underweight, overweight and obesity in adults (Table 3). BMI values are age-independent, the same for both sexes and do not take into consideration differences in body proportions (World Health Organisation, 2006). BMI generally correlates highly with adiposity but the calculation may not correspond to the same degree of adiposity in different populations such as in athletes and the very tall and very short (Freeman, Power, & Rodgers, 1995). A recent study of 25,241 diagnosed type 2 diabetic patients and 50,482 matched controls found that the BMI values were not only 'strongly and independently associated with the risk of being diagnosed with type 2 diabetes', but also that the magnitude of this positive association was larger for higher BMI values (Ganz et al., 2014).

Table 3. WHO international classification of BMI.
Adult underweight, overweight and obesity according to BMI (World Health Organisation, 2006).

Classification	BMI	Risk of comorbidities
Underweight	< 18.5	Low (but risk of other clinical problems increased)
Normal range	18.5 – 24.9	Average
Overweight:	≥ 25.0	
Preobese	25.0 – 29.9	Increased
Obese class I	30.0 – 34.9	Moderate
Obese class II	35.0 – 39.0	Severe
Obese class III	≥ 40.0	Very severe

The Nurses' Health Study in 1997, after analysing data from 43,581 participants, concluded that BMI, waist circumference, and waist to hip ratio, were all independent determinants of metabolic syndrome risk throughout their ranges of values (Carey et al., 1997). It is well established that BMI correlates closely with weight, clinical outcomes and mortality (Al Asfoor, Al Lawati, & Mohammed, 1999; Bastien, Poirier, Lemieux, & Despres, 2014; Carey et al., 1997; Deurenberg & Yap, 1999; Wang et al., 2009; World Health Organisation, 1995, 2000a)

An excess of abdominal fat is closely associated with metabolic risk factors (Scott M. Grundy, 2004), and in fact, increases in waist circumference have been shown to be associated with increased risk factors for cardiovascular disease (Han, van Leer, Seidell, & Lean, 1995; Pouliot et al., 1994). From the clinical perspective, visceral adipose tissue is known to generate diabetogenic substances and thus may be more informative than total fat for diagnostic evaluation (Vazquez, Duval, Jacobs, & Silventoinen, 2007). An established way to estimate abdominal obesity in clinical practice is to measure waist circumference.

The waist circumference is taken half way between the top of the iliac crest and the bottom ribs, at the level of the umbilicus. The tape should sit parallel to the floor and lie snug on the skin without compression (Pouliot et al., 1994; Wang et al., 2009). Loman et al. (1991) determined the measurement error of the waist measurement in studies of adolescents. They calculated the average technical error of the waist circumference measurement to be 1.31 cm from intra-measurer error and 1.56 cm from inter-measurer error, considered not clinically significant. Although it is arguable on how to determine body fat percentage accurately, body fat greater than 25% in men and 35% in women has been used by some authors to determine obesity (Scott M. Grundy, 2004).

Correlations between waist circumference and body mass index have been observed in the past (Abbasi, Blasey, & Reaven, 2013; Yu et al., 2015). A recent examination of data collected in the USA National Health and Nutrition Examination Survey observed strong correlations between BMI and waist measurements ($r = \sim 0.9$) (Freedman & Ford, 2015).

2.6.6.1 Homeostatic model assessment.

The homeostatic model assessment (HOMA) is a calculation used to assess beta cell function ($HOMA_B$ = 20 x fasting insulin (microU/L)/ fasting glucose (nmol/L) – 3.5) and insulin resistance ($HOMA_{IR}$ = fasting insulin (microU/L) x fasting glucose (nmol/L)/ 22.5) from a patients fasting blood glucose and insulin measures Wallace et al., 2004). Turner et al. (1979) developed the HOMA mathematical model based on the negative feedback loop between beta cell function

and the liver, and the influence this feedback has on blood glucose regulation and insulin sensitivity.

A 2001 study investigated the accuracy and clinical usefulness of HOMA as an index of insulin resistance, in 55 type 2 diabetic patients undergoing diet and exercise therapy (Katsuki et al., 2001). Insulin resistance was assessed by hyperinsulinemic euglycaemic clamp, a two hour peripheral vein infusion of glucose, that measures the necessary amount of glucose required to compensate for increased insulin levels, without causing hypoglycaemia. This technique is mainly utilized in research. The authors found significant correlations between $HOMA_{IR}$ and hyperinsulinemic euglycaemic clamp ($r = -0.617$, $p < 0.0001$) and deemed the HOMA calculations a useful method for insulin resistance diagnosis (Katsuki et al., 2001). HOMA's usefulness as a clinical and epidemiological tool has been demonstrated in a number of other studies (Katsuki et al., 2001; Levy, Matthews, & Hermans, 1998; Matthews et al., 1985; Wallace, Levy, & Matthews, 2004).

2.7 Clinical Potential for FCB-DM

Darkfield microscopic analysis of fresh capillary blood, like many medical screening devices, has its strengths and limitations (Vitetta et al., 2005). This research aims to improve health practitioner's understanding of FCB-DM and to reveal if it has potential to support patient health outcomes. As discussed earlier, darkfield microscopy enables the clinician to clearly observe leukocytes and erythrocytes in unstained blood. Reports of clinical observations outline some of the potential strengths of FCB-DM in patient care including observations of myelodysplasia, abnormal lymphocytes and red cell oxidation (Mannion, Barbiellini, & Reilly, 2010; Reeve & Reilly, 2011; W. Reilly, 2009; Reilly & Mannion, 2009; Reilly et al., 2010).

This study focussed on investigating the validity of four specific FCB-DM variables: platelets, fibrin, chylomicron remnants and the differential white blood cell count. The following sections provide background information in these areas.

2.8 Fresh capillary blood analysis variables in obesity.

Though there is a large amount of literature and research in the area of

metabolic syndrome and insulin resistance diagnosis and treatment, there is little research investigating the use of FCB-DM as a tool in monitoring these disease states. In theory, FCB-DM offers a cost effective and non-invasive point of care patient screening and monitoring tool for patients with metabolic disorders (Reilly et al., 2010; Vitetta et al., 2005).

2.8.1 Platelet aggregation.

German microscopic anatomist, Max Johann Sigismund Schultze published the first description of platelets in 1865, recognising them as normal constituents (Brewer, 2006) of the blood. Platelets are non-nucleate cytoplasmic fragments derived from megakaryocytes, which are involved in haemostasis (Dacie & Lewis, 1991, p. 284; Kumar & Clarke, 2005, p. 466). The average lifespan of a platelet in the body is 8 to 10 days (Harker et al., 2000), and the normal platelet count in humans ranges from 150 $x10^9$ to 400 $x10^9$ per litre (Brass, 2010; Dacie & Lewis, 1991, p. 58; Daly, 2011).



Figure 4. Electron micrograph images of platelets.
Scanning electron micrograph of resting (left) and activated (right) platelets. (http://pixgood.com/activated-platelet.html, retrieved on 28/12/2014)

If the blood vessel endothelial layer is disrupted through injury, basement membrane structures such as collagen and microfibrils are exposed (Dacie & Lewis, 1991 pg 269). Adhesion receptors at the site of injury bind von Willebrand factor and collagen, which initiate platelet adhesion and activation (Andrews & Berndt, 2004). The platelet adhesion leads to the development of a haemostatic plug over the injured site, a process referred to as primary haemostasis. Once activated, platelets change

from a disk shape to a sphere and develop pseudopod projections (Barkalow et al., 2003; Gravel, Zheng, Sims, & Dixon, 1994) (Figure 4).

Brecher et al. (1950) observed platelets under DM in the 1950's (Figure 5). From their research they concluded that the variety of platelet shapes depended on the thickness of the preparation, surface property of the glass and concentration of coagulant as well as possibly the pH and temperature. In FCB-DM platelets can be seen as irregularly shaped, dense blue-white grainy structures ranging greatly in size from 2 µm to upward of 80 µm (Metagenics, 2010, pp. 60-62; Petric, 2009, pp. 17-18). Platelet activation begins as soon as blood is withdrawn from the fingertip. The classic marker of platelet hyperactivity is 'spontaneous' aggregation (Brass, 2010), as observed in laboratory controlled conditions. The degree and speed of aggregation can be used as an indicator of the reactivity of the platelets in vivo and can also be an indicator of cardiovascular risk (Petric, 2009). There is increasing evidence of the role of platelets in thrombotic diseases and vascular processes such as inflammation and atherogenesis (Yip, Shen, Berndt, & Andrews, 2005).

2.8.2 Fibrin.

Primary haemostasis is defined as the formation of the platelet plug, which seals off bleeding at the site of injury. Platelets express phosphatidylserine on their surface which encourages the formation of coagulation factor complexes, amplifying fibrin formation (Kumar & Clarke, 2005, pp. 466-467). Occurring simultaneously to primary haemostasis, secondary haemostasis regulates the deposition and removal of fibrin in the body (Dacie & Lewis, 1991, p. 273). Several coagulation factors are involved in the clotting process, most of which are formed in the liver and, in general, are in an active state in the blood (Silbernagel & Despopoulos, 2009, p. 102). Activation of these proteins occurs in a cascade of events initiated by an exogenous or endogenous trigger. Prothrombin is converted to thrombin, which in turn activates a number of acute phase proteins including the conversion of fibrinogen to fibrin. Fibrin enmeshes the platelet aggregates at the site of injury, and stabilizes the haemostatic plug (Hoffbrand, 2006, p. 320).

This process is carefully regulated by the body to prevent excessive clotting

and occlusion of blood vessels. Fibrinolysis is mediated by plasmin at the site of injury limiting the size of the evolving thrombus (Hoffbrand, 2006, p. 326).

Various coagulation factors including fibrin and fibrinogen modulate the body's inflammatory response by affecting leukocyte migration and cytokine production. Confining inflammation or infection is a complex process ensuring elimination of dangerous stimuli to return the body to homeostasis (Jennewein et al., 2011). A number of inflammatory disease states such as rheumatoid arthritis, multiple sclerosis and obesity, involve a disruption to the inflammatory and coagulation processes in the body (Jennewein et al., 2011). Plasminogen activator inhibitor 1 (PAI-1) levels rise in response to inflammation while inflammatory mediators such as IL-6 increase platelet count and reactivity. This leads to an amplification of the coagulation process and impaired ability to remove the thrombus (Esmon, 2004).

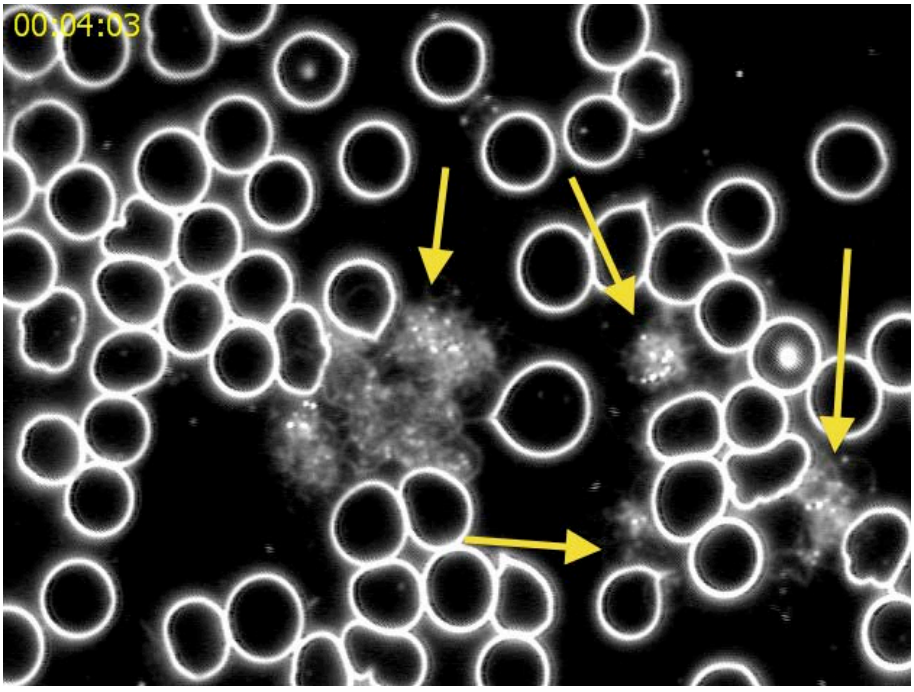


Figure 5. Platelet clusters surrounded by erythrocytes as seen by FCB-DM. Photo sourced from research data.

A 2003 study of 1276 people randomly selected from Asia, America and Europe found that people with metabolic syndrome had significantly higher PAI-1 than people without metabolic syndrome (24.2 versus 14.6 U/mL; $p < 0.001$) and the time it took to lyse a blood clot in vitro in samples from people with metabolic syndrome was significantly longer than those without (Anand et al., 2003). People

with metabolic syndrome were also found to have higher concentrations of fibrinogen (E. S. Ford, 2003).

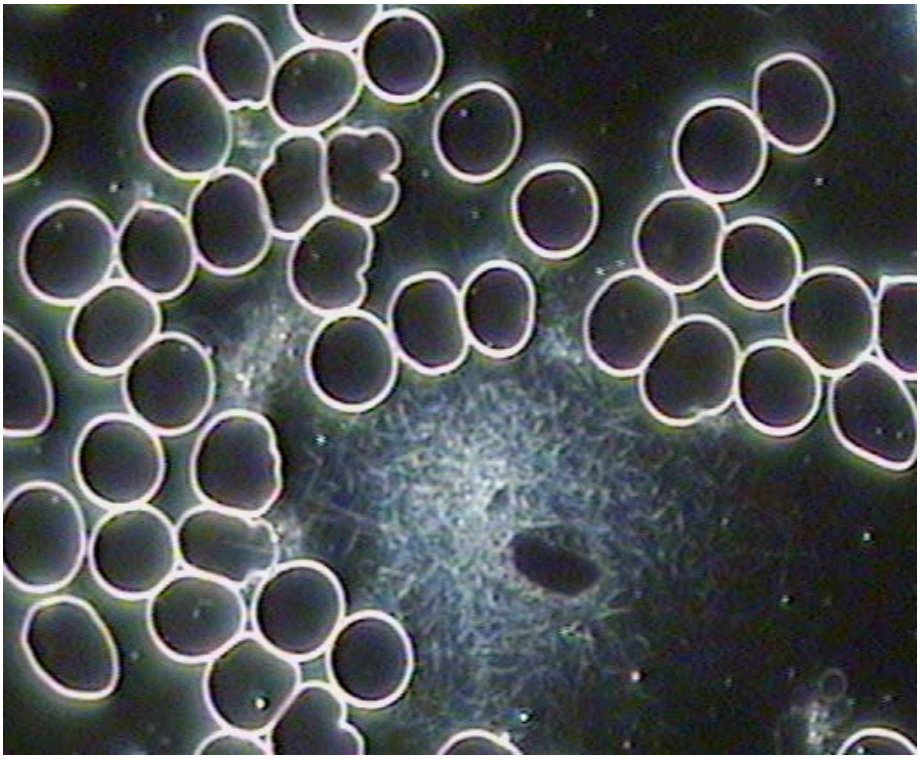


Figure 6. Fibrin cluster as seen by FCB-DM.
Photo sourced from research data.

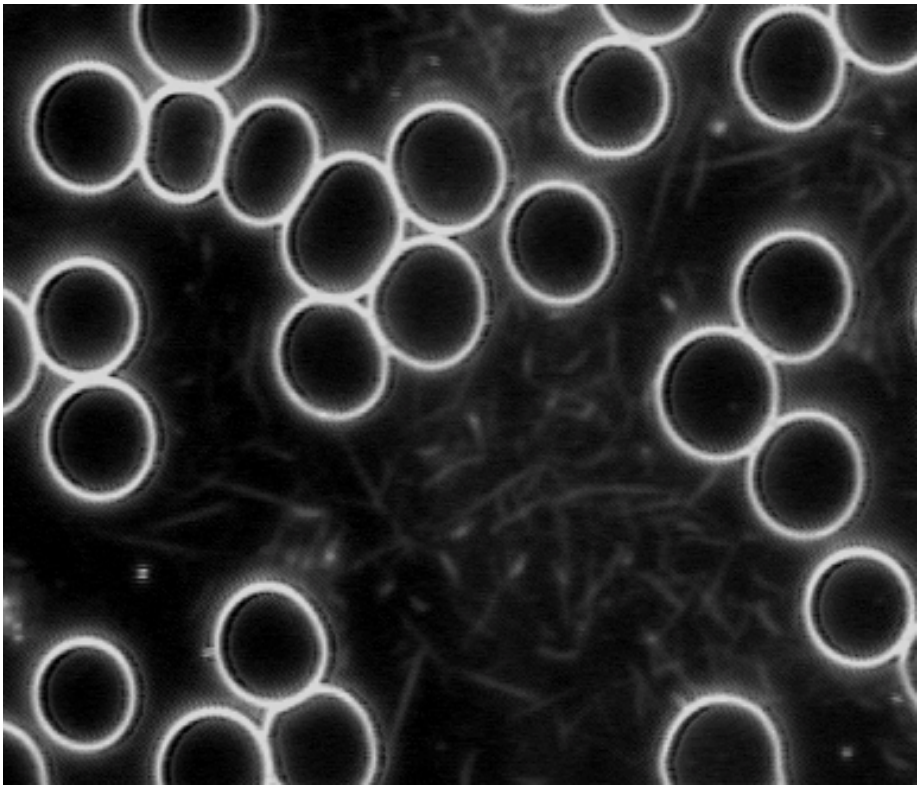


Figure 7. Diffuse fibrin as seen by FCB-DM.
Photo sourced from research data.

Fibrin deposits within inflamed tissues are not just a reflection of a disease process but actively contribute to disease pathogenesis (Raghu & Flick, 2011). One mechanism that has been demonstrated to directly link fibrin to the regulation of inflammation is the ability of fibrin to serve as a ligand for cell-surface receptors, particularly integrins (Koh, Han, & Quon, 2005; Raghu & Flick, 2011). Therefore they are useful blood markers for monitoring inflammation.

Fibrin can be observed in FCB-DM as clusters of white fibrous particles (Figure 6) called fibrin clusters, or these same particles spread throughout the sample, as a 'disorganised spider web' (Rubik, 2010) termed diffuse fibrin (Figure 7) (Metagenics, 2010, pp. 66-69).

2.8.3 Chylomicron remnant clearance.

Chylomicrons are lipoproteins that transport fats and fat-soluble vitamins from the intestine to the lymphatic circulation, then into the blood for use by body cells. Lingual lipases in the lumen of the stomach and pancreatic lipases in the small intestine break down dietary fats into small particles. These particles are then absorbed into the intestinal mucosal cells and are resynthesized into chylomicrons (Gropper et al., 2005, pp. 137-140; Hultin & Olivecrona, 1998).

Chylomicron remnants are what remain in the blood after lipoprotein lipase removes part of the triglycerides from the chylomicron (Bierman, Gordis, & Hamlin, 1962; Elkes et al., 1939; Flatt, Ravussin, Acheson, & Jequier, 1985). Chylomicron remnants can be clearly observed in FCB-DM and have been observed in postprandial blood samples since the 1930's (Elkes et al., 1939; Frazer & Walsh, 1933). Early research in 1936 found that alterations to the particles seen in blood under DM, only occurred in relation to ingestion of fatty food (Elkes et al., 1939). A marked increase in chylomicron remnants in blood samples one and a half hours after ingestion of a meal containing fat was reported, with levels normalising by four and a half hours postprandial. Interestingly, a small rise ('initial rise') in chylomicron remnants 25-30 min postprandial was observed and this was postulated to be due to the residual fatty acids in the blood stream from the previous meal (Elkes et al., 1939). More recently, it has been observed that the consumption of a fatty meal causes a rise in

triacylglyceride-rich lipoprotein particles and an increase in nuclear factor kappaB (NF-kappaB) which in turn promotes the transcription of pro-inflammatory products (Delgado-Lista et al., 2008; Duttaroy, 2005).

Chylomicron remnants are visible in FCB-DM as dense white particles around 1 µm in diameter (Metagenics, 2010, p. 100)(Figure 8) that appear to oscillate in the blood sample most likely due to Brownian motion. Chylomicron remnants peak in normal samples at 1.5 to 2.5 hours after food, and the peak reported value averaged at 125 to 175 chylomicron remnants per viewing field at a 12.5x objective with a stop 1/16 inch squared. (R. R. Cooper & Lusk, 1942; Elkes et al., 1939).

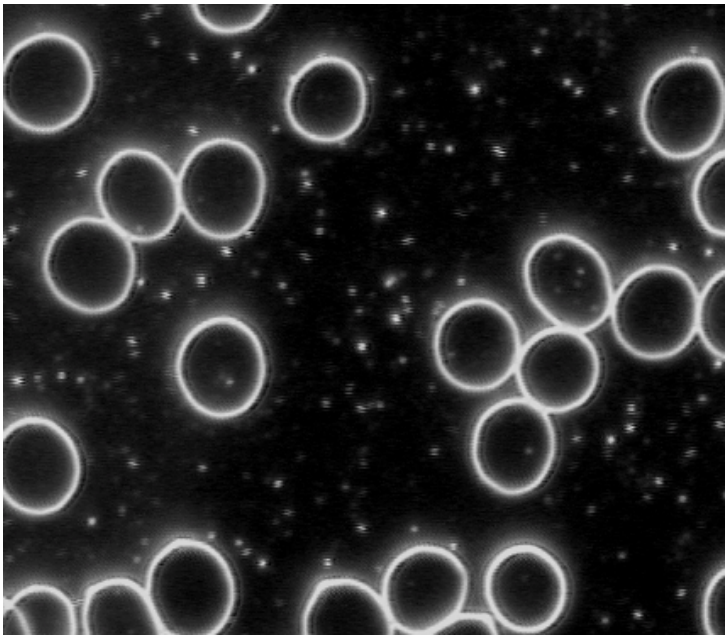


Figure 8. Chylomicron remnants as seen by FCB-DM.
Chylomicron remnants appear as small white dots in the background as seen by FCB-DM. Photo sourced from research data.

Darkfield microscopic analysis of fresh capillary blood is not able to demonstrate or measure blood glucose or insulin levels, but another of the proposed uses as a screening tool for metabolic syndrome risk, is in the observation of chylomicron remnant presence. Accumulating evidence indicates a link between obesity, insulin resistance and dyslipidaemia (Abbott et al., 1987; Bansal et al., 2007; Chahil & Ginsberg, 2006; Frayn, 1993; Garg, 1996; U. J. Jung & Choi, 2014; Tomkin & Owens, 1994; Zammit, 1996). Chylomicrons and chylomicron remnants have, until recently, been thought to be the major lipoproteins in the postprandial

hyperlipidaemic patient but new analytical methods confirm the major portion of triglycerides that increase in the postprandial state are triglyceride rich lipoprotein remnants (Nakajima et al., 2011). Fatty acids released by adipose tissue result in increased delivery of fatty acids to the liver, causing an increased synthesis of very-low-density-lipoprotein (VLDL). This increased synthesis of VLDL inhibits lipolysis of chylomicrons, promoting hypertriglyceridaemia (U. J. Jung & Choi, 2014).

Clinical observations indicate that chylomicron remnant presence in capillary blood, when seen through DM, is possibly affected by how glucose is utilisd by the patient as well as a reflection of the patient's triglyceride levels (Reilly, 2011).

2.8.4 Differential white blood cell counts.

White blood cells or leukocytes are nucleated cells present in varying concentrations in human blood. They can be classified according to their morphological appearance and immunological function (Witko-Sarsat, Rieu, Descamps-Latscha, Lesavre, & Halbwachs-Mecarelli, 2000).

Manufacture and development of leukocytes occurs in the bone marrow. Only mature white blood cells are found in healthy peripheral blood. During childhood and adulthood, the bone marrow is the primary site of haemopoiesis, though the liver and spleen can resume their foetal haemopoietic function if required (Hoffbrand, 2006, p. 111). Haemopoiesis begins in the bone marrow from pluripotent stem cells that are self-renewing and can give rise to different cell lineages. These cells are able to repopulate the bone marrow after damage from, for example, chemotherapy or radiation (Hoffbrand, 2006, p. 112).

Pluripotent stem cells develop two types of progenitor cells, common myeloid and common lymphoid. Common myeloid cells, termed colony forming unit are the earliest detectable mixed myeloid precursors from which granulocytes, erythrocytes, monocytes and megakaryocytes develop (Haurie, Dale, & Mackey, 1998). Lymphocytes differentiate from the common lymphoid precursor.

Haemopoietic growth factors are glycoprotein hormones that regulate

proliferation and differentiation of the progenitor cells and the function of mature blood cells (Hoffbrand, 2006, p. 6). These include cytokines like IL-6 and TNF that act on stromal cells, stem cell factor (SCF), Fms-like tyrosine kinase 3 ligand (FLT3-L) and vascular endothelial growth factor (VEGF) that in turn act on pluripotent cells among others (Hoffbrand, 2006). Growth factors are mainly induced by stromal cells except for erythropoietin, which is mainly synthesised in the kidney and thrombopoietin made mostly in the liver (Hoffbrand, 2006, p. 112).

2.8.4.1 Neutrophils.

Various white blood cells present in the blood at any one time have a variety of different functions. Granulocytes are phagocytic white blood cells involved in immunological surveillance. These cells contain a high concentration of granules, which contain proteases and peroxides, and are readily distinguishable under dark field microscopy. Neutrophils, eosinophils and basophils are all granulocytes (Hoffbrand, 2006, p. 110).

Neutrophils are the most numerous of leukocytes in a normal blood sample, making up 40-65% of the total white blood cell count. These cells are mobilised within a few hours of an immune challenge, circulate in the blood for only 6-8 hours before migrating into the tissues (Borregaard, 2010; Hoffbrand, 2006). They are generated in large numbers in the bone marrow, from stem cells that differentiate and proliferate into mature neutrophils that contain protein granules. Neutrophils have multidirectional motility and have non-specific phagocytic function (Hoffbrand, 2006, p. 110). Neutrophil granules are classified into three groups: primary azurophil granules (myeloperoxidase); secondary specific granules (lactoferrin) and tertiary gelatinase granules (Borregaard, 2010).

Under darkfield microscopes neutrophils appear bright and silver-white. They are approximately 10-16 μm or one and a half to two times as large as erythrocytes (Figure 9). The granules in the cytoplasm are clearly visible, and typically between 1 and 4 lobes are seen as dark segments (Metagenics, 2010).

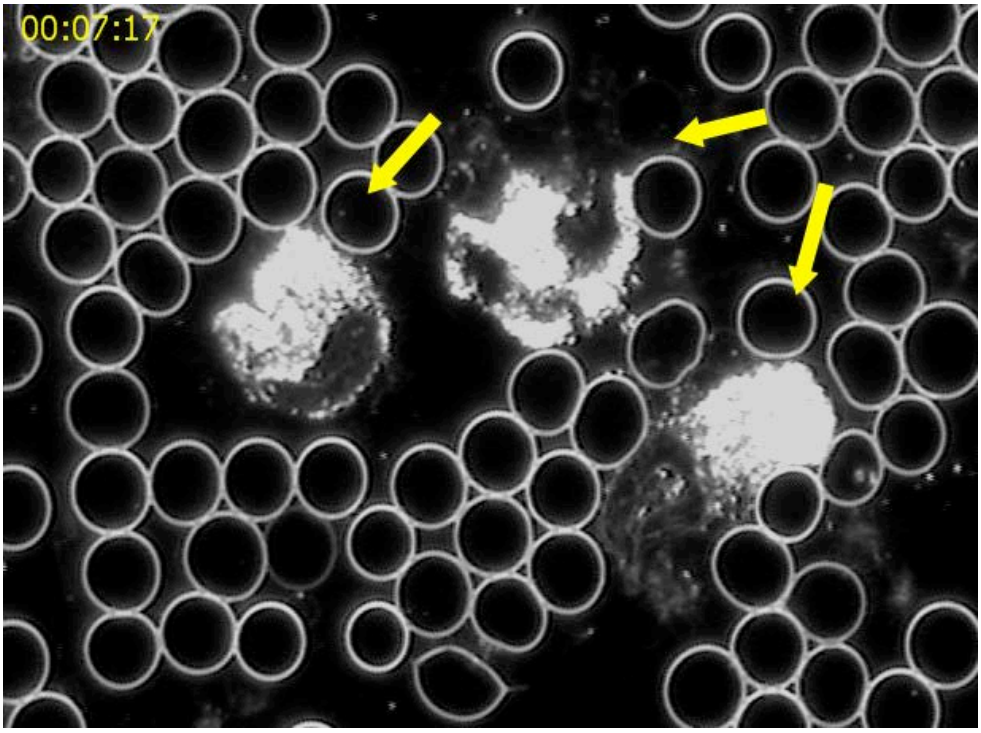


Figure 9. Neutrophils as seen by FCB-DM.
Photo sourced from research data.

The phagocytic neutrophil is attracted to bacteria or the site of inflammation by chemotactic substances released from damaged tissue, complement components or by the interaction of leukocyte adhesion molecules with ligands on the damaged tissue (Hoffbrand, 2006, p. 114). These adhesion molecules are glycoproteins that interact with ligands on the target cells, and mediate attachment of leukocytes and platelets to the extracellular matrix, endothelium, each other and other surfaces (Hoffbrand, 2006, p. 114). There are three groups of adhesion molecules, immunoglobulins, selectins and integrins. Immunoglobulins react with antigens and antigen dependent surface adhesion molecules; selectins enable leukocyte and platelet adhesion to the endothelium during inflammation and coagulation, and integrins are involved in cell adhesion to the extracellular matrix such as in collagen formation in wound healing (Hoffbrand, 2006, p. 114). The expression of glycoprotein adhesion molecules can be modified by intracellular and extracellular factors and their function can be quantitative or functional. TNF, IL-1, IFN-gamma, T-cell activation and viral infections up-regulate these molecules.

It was believed that neutrophils were pro-inflammatory and that their presence was solely damaging to tissue (Anderson, Brown, & Harken, 1991), but more recent

research has exposed the benefit of these clever cells (Amulic, Cazalet, Hayes, Metzler, & Zychlinsky, 2012). As a key component in the inflammatory response, neutrophils also contribute to the recruitment and activation of antigen presenting cells by generating chemotactic signals that attract monocytes, dendritic cells and influence macrophage differentiation (Nathan, 2006). The dual function of immune surveillance and *in situ* elimination of microorganisms or cellular debris requires neutrophils to rapidly transform from a non-adherent circulating state to an adherent state, allowing ease of migration into the tissues (Witko-Sarsat et al., 2000). Once firm adhesion of the neutrophil to the activated endothelium is established, two pathways for trans-endothelial migration can be taken: trans-cellular where the neutrophils penetrate the individual endothelial cell, or para-cellular, where the neutrophils squeeze between the endothelial cells (Borregaard, 2010).

Once inside the cell phagocytic digestion occurs via both oxygen and non-oxygen dependent pathways. Oxygen dependent hydrogen peroxide in neutrophils reacts with myeloperoxidase and intracellular halide to kill bacteria. This production of reactive oxygen intermediates is known as the respiratory burst or oxidative burst (Nathan, 2006). The non-oxidative microbicidal proteins such as lysozyme can act alongside the oxygen dependent pathway or independently in reaction to the fall in pH (Hoffbrand, 2006, p. 115). In addition, the iron binding protein lactoferrin is present in neutrophil granules and is bacteriostatic, depriving the bacteria of iron and creating free radicals (Ambruso & Johnston, 1981).

Neutrophil numbers can change rapidly in response to infection or tissue damage as large numbers of band and segmented neutrophils are held in the bone marrow as a ‘reserve’ pool (Hoffbrand, 2006, pp. 111-112). This pool in normal healthy blood holds 10-15 times the number of granulocytes as in peripheral blood, ready for release if required. Examples of causes of neutropenia and neutrophilia can be seen in Appendix F.

2.8.4.2 Lymphocytes.

Lymphocytes constitute the body’s adaptive immunity and are the second most abundant white blood cell in healthy human blood accounting for 20-35% of a

normal white blood cell count (Bain, 2005, p. 725; Metagenics, 2010).

Lymphopoiesis occurs in the bone marrow from common lymphoid progenitor cells before differentiating into distinct types. B lymphocytes mature in the bone marrow and circulate in peripheral blood whereas T lymphocytes develop from cells that migrate to the thymus where they differentiate into mature cells through the migration from the cortex to the medulla (Hoffbrand, 2006, p. 127). During this process of migration in the thymus, self-reactive T cells are deleted and T cells that display some specificity for the host human leukocyte antigen molecules (HLA) are selected.

There are two main types of T cells – helper T cells that express CD4 and cytotoxic T cells that express CD8. Natural Killer cells are cytotoxic CD8 cells that lack the T cell receptor. They kill target cells that have a low level of expression of HLA class I molecules as occurs for instance in viral infection or on a malignant cell (Hoffbrand, 2006, p. 129), as part of cell mediated immunity.

Mature B lymphocytes, or plasma cells, contain membrane bound immunoglobulin receptors that produce antibodies when the cell is activated by contact with an antigen as part of humoral immunity (Bain, 2005, p. 725). Following maturation, lymphocytes continually migrate from the peripheral blood into lymphoid organs such as the spleen, lymph nodes or bone marrow via post capillary venules (Alberts, Johnson, & Lewis, 2002).

Lymphocytes can be clearly seen in FCB-DM (Figure 10) but the technique is unable to distinguish between B and T lymphocytes as the cells are morphologically indistinguishable prior to activation (Bain, 2005). They appear as dull cells with grey cytoplasm and a large dark nucleus that occupies 70-80% of the cell (Metagenics, 2010).

The total lymphocyte percentage in the white cell differential count can indicate immune function and viral exposure. Lymphocytosis or raised lymphocyte count can be caused by acute or chronic infections including Epstein-Bar virus, cytomegalovirus, infectious mononucleosis, mumps, infectious hepatitis, tuberculosis,

toxoplasmosis, syphilis and HIV (Hoffbrand, 2006, pp. 135-137). Other causes can include chronic lymphoid leukaemia, acute lymphoblastic leukaemia, non-Hodgkins lymphoma, thyrotoxicosis, Addison's disease and autoimmune diseases. It is normal for children up to 12 years of age, to have an elevated lymphocyte count of around 40% (Denny et al., 1992).

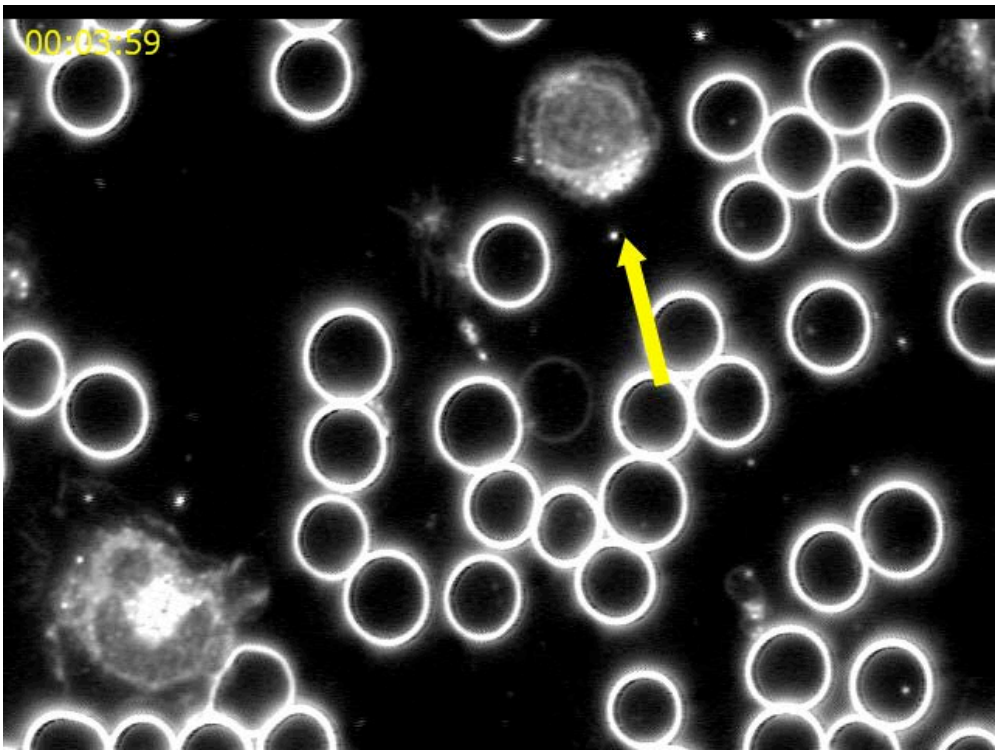


Figure 10. Monocyte and lymphocyte as seen by FCB-DM.
Monocyte (bottom left) and lymphocyte (Centre top with arrow) as seen in FCB-DM. Photo sourced from research data.

Lymphocytopenia can be seen mainly in association with several congenital diseases of the immune system or following treatment with corticosteroids, antineoplastic agents, or radiation (Bain, 2005, p. 726; Blumenreich, 1990).

A case study was presented at the 2011 Molecular and Experimental Pathology Society of Australasia's annual conference demonstrating the usefulness of FCB-DM in lymphocyte identification (Appendix C). A routine FCB-DM was performed and lymphocytosis, monocytosis and blast cells were observed. The lymphocytes were of abnormal morphology, larger than standard with hair like projections. The patient was referred to her GP for further investigations. Automated pathology counts and blood film confirmed a previously unseen diagnosis of hairy

cell leukaemia (Reeve & Reilly, 2011).

2.8.4.3 Monocytes.

Monocytes usually make up 3-8% of the total white cell differential count in normal blood and are the largest of the leukocytes (Blumenreich, 1990). They develop from myeloid stem cells and are released into the circulation for 1-3 days before migrating into tissues (Bain, 2005, p. 725). Monocytes are important accessory and regulatory cells in humoral and cellular immunity (Rothenberg & Hogan, 2006).

Monocytes are large cells with a central oval or kidney shaped nucleus with clumped chromatin (Hoffbrand, 2006, p. 111). In FCB-DM they appear as dull white-grey cells, 1-3 times the size of a red blood cell with a nucleus that occupies 50% of the cell (Metagenics, 2010) (Figure 10).

The collective term for circulating monocytes in the blood and macrophages in tissues is the mononuclear phagocytic system (Wu et al., 2011). When monocytes migrate into tissues they differentiate into highly mobile macrophages or fixed cells such as Kupffer cells in the hepatic sinus, Langerhans cells in the skin, dendritic cells, or microglia in the brain (Ullmann, 2001, p. 96).

Monocytes-macrophages phagocytose micro-organisms or cellular debris, play a role in the inflammatory reaction and participate in fibrinolysis by secreting plasminogen activators (Bain, 2005, p. 725; Zuo & Rothenberg, 2007). They secrete TNF and IL-1 and IL-12 that potentiate B and T lymphocytes.

Undifferentiated monocytes reside in the spleen in higher numbers than in circulation. In response to injury, splenic monocytes increase their motility, exit the spleen, accumulate in injured tissue, and participate in wound healing (Young, Lowe, Stevens, & Heath, 2006).

Monocytosis can follow chronic infectious disorders (tuberculosis, brucellosis), rheumatic diseases (lupus, rheumatoid arthritis), chronic inflammatory bowel disease, and some malignant processes (Hodgkin's and non-Hodgkin's

lymphoma) (Bain, 2005; Blumenreich, 1990).

2.8.4.4 Eosinophils.

Eosinophils are the fourth most abundant type of white blood cell, accounting for 0-4% of the total white cell differential count in a normal human blood sample (Young et al., 2006). They differentiate from myeloid precursor cells in the bone marrow. Three cytokines (IL-3, IL-5, and GM-CSF) are important regulators of eosinophil development under the instructive signals from a number of transcription factors, namely GATA-1, PU.1, and C/EBPs (Rothenberg & Hogan, 2006).

Eosinophils are multifunctional leukocytes involved in initiation and propagation of diverse inflammatory processes including parasitic helminth, bacterial and viral infections, tissue injury, tumour immunity, and allergic diseases (Hogan et al., 2008; Rothenberg & Hogan, 2006). Eosinophils have also been shown to respond to foreign protein substances such as allergic antigens, fungal infections, some drugs and exposure to certain chemicals (Hoffbrand, 2006, p. 111).

These cells largely reside in the tissues where they can live for up to two weeks. The majority of eosinophils are found in the lamina propria of the gastrointestinal tract except the oesophagus, thus increased presence in the blood is acknowledged as clinically significant (Hogan et al., 2008; Zuo & Rothenberg, 2007).

They enter inflammatory exudates and have a special role in fibrin removal in inflammation (Hoffbrand, 2006, p. 111). Eosinophils can serve as major effector cells killing pathogens but also inducing tissue damage of healthy cells by releasing toxic granule proteins and lipid mediators (Hogan et al., 2008). They produce cytokines that contribute to their pro-inflammatory functions.

Alongside their role in acute inflammation, eosinophils are recognised as immunomodulatory cells interacting with B and T lymphocytes (Bandeira-Melo & Weller, 2005). Eosinophils can act as antigen presenting cells and activate antigen-specific responses.

New research has shown that eosinophils can migrate to adipose tissue to maintain glucose homeostasis, increasing comprehension of the links between immune and metabolic systems (Wu et al., 2011).

Eosinophils are suppressed by cortisol, thus numbers are suppressed with stress and concentrations can rise throughout the day. Increased numbers are also found after exercise and numbers are also affected by menstrual patterns in women (Davis & Hulit, 1949; Metagenics, 2010).

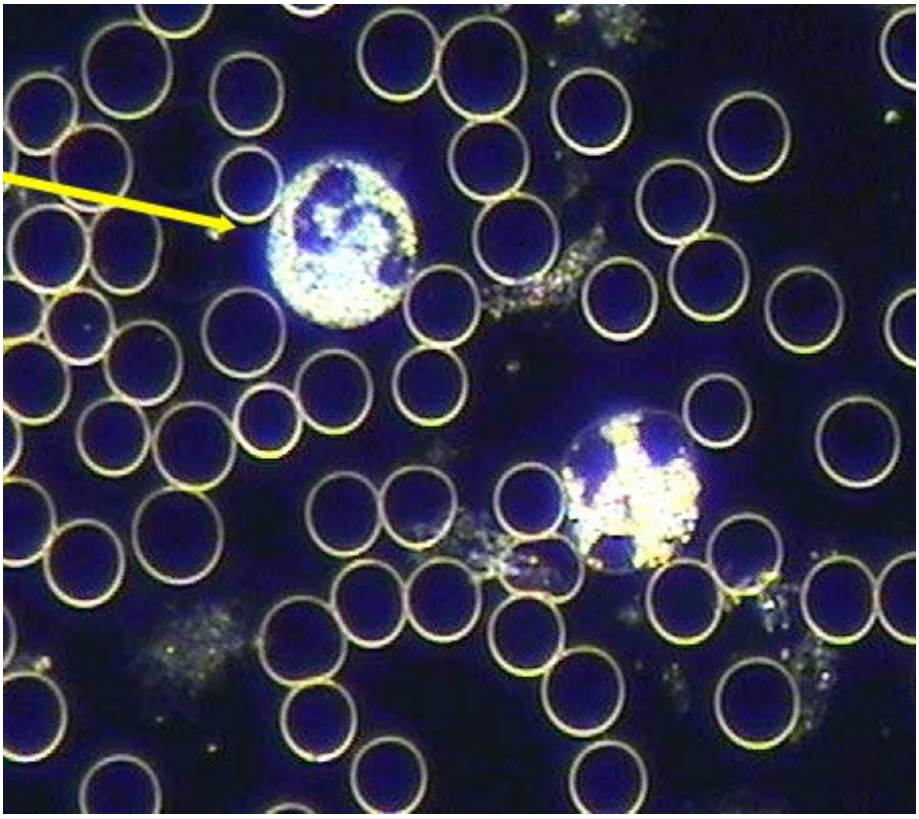


Figure 11. Neutrophil and eosinophil as seen by FCB-DM.
Neutrophil (top with arrow) and eosinophil (centre right) as seen in FCB-DM. Photo sourced from research data.

Increased levels of circulating and tissue eosinophils are characteristic features of a number of pathological conditions for example parasitic infections, local allergic reactions such as asthma, rhinitis and eczema, and urticarial (Conroy et al., 1997). Recovery from acute infection, drug sensitivities, polyarteritis nodosa and vasculitis are also causes of increased concentrations, as are Hodgkins lymphoma, chronic eosinophilic leukaemia and pulmonary syndromes (Hoffbrand, 2006, p. 121).

Eosinophils are differentiated from other granulocytes in FCB-DM by their golden colour and cytoplasmic granules that are larger than a neutrophil's (Metagenics, 2010) (Figure 11).

2.8.4.5 Basophils.

Basophils normally have the lowest presence of all leukocytes in the blood, making up less than 1% of the total white cell differential count. They have large granules, which contain heparin and histamine (Hoffbrand, 2006, p. 111) and become mast cells in the tissue.

Recent research has demonstrated basophils' role in bridging innate and adaptive immunity through responding to various stimuli mediated by both IgE-dependant and IgE-independent mechanisms (Cromheecke, Nguyen, & Huston, 2014). The degranulation of basophils is associated with histamine release, as well as rapid generation of LTC4, IL-4, IL-6, IL-13 and Th2 cytokines (Cromheecke et al., 2014; Siracusa, Perrigoue, Comeau, & Artis, 2010).

Basophils have been observed in FCB-DM using blood from patients previously diagnosed by a physician with environmental allergies and states of chronic immune suppression (Witko-Sarsat et al., 2000).

2.8.4.6 Differential white blood cell count.

Differential white blood cell counts are routinely performed as part of a full blood count in orthodox pathology (Barnes et al., 2005) and indicate the number of different white blood cell types in a sample of blood. This test provides quantitative information about blood particles, and aids diagnosis of illness, infection and inflammation. These measurements are useful in monitoring and treating immune mediated diseases (autoimmunity, inflammatory conditions, HIV), infection, allergies, diabetes, cardiovascular risk, illness recovery and cancer treatments (Brown, Giles, & Croft, 2001; Hoffbrand, 2006, p. 109).

Manual rapid impression scanning has been investigated as a time saving, less labour intensive alternative to the manual differential white cell count, but it has been

shown to be less reliable (Arkin et al., 1987) and suggested to be used only in conjunction with automated counts, to increase the detection of abnormal cells where required.

There is no requirement for differential staining in FCB-DM, as leukocytes exhibit colour due to the differential absorption of white light by cytoplasmic granules in the different cell types (Rottenfusser, Wilson, & Davidson, 2014)

2.8.4.7 Differential white blood cell counts – manual v's automated.

Investigating the accuracy between manual and automated differential white cell counts, Pierre (1985) found manual counting lacked sensitivity and specificity compared to automated differential counts. Numerous studies in the 2000's indicated that automated differential counts were as accurate, if not more accurate as the manual count (Buttarello & Plebani, 2008; Crissey & Denenholz, 1984; Jo et al., 2011; Osei-Bimpong et al., 2009; Pierre, 2002). A number of these articles do still stress the importance of maintaining blood film and manual analysis techniques of blood to support accurate identification of morphologic abnormalities in appropriate blood samples (Jo et al., 2011; Pierre, 2002).

A 2002 paediatric oncology study observed 111 specimens to determine the efficacy of automated absolute neutrophil counts in comparison to manual counts during chemotherapy monitoring (Parham et al., 2002). Automated count values ranged from .16-14.2 $x10^9$/L (median 2.6 $x10^9$/L) compared with manual count values .24-13.9 $x10^9$/L (median 3.0 $x10^9$/L). The authors concluded that the automated counts were adequate for determining suitability for chemotherapy and monitoring patients undergoing chemotherapy.

2.9 Summary

Darkfield microscopic analysis of fresh capillary blood is utilised as a screening tool in the complementary health care arena. The blood components such as erythrocytes, leukocytes, platelets, lipid and clotting particles, are readily visualised and quantifiable in FCB-DM. However there is a scarcity of scientific research based evidence pertaining to the accuracy of FCB-DM as a valid and reliable screening tool.

The literature outlines clearly the link between visceral adiposity and metabolic syndrome. Clinical evidence suggests FCB-DM can provide practitioners with information, at point of care, relating to patients' immune function, lipid metabolism and inflammatory responses. Examination of the relationship between FCB-DM and the acknowledged standard, venous blood measures, would expand knowledge of the technique and its validity and reliability as a screening tool in the clinical setting.

3 Methodology

To investigate the identified hypotheses, this Master of Science thesis included two studies that utilised quantitative research methods:

1. The metabolic syndrome study focussed on investigating hypotheses A, B and C. This study investigated the validity of FCB-DM variables compared to fasting venous blood pathology measures. Participants were specifically recruited for this project in Brisbane (n=51) and Lismore (n=19).
2. The differential white blood cell study focussed on investigating hypothesis D. This study collapsed 74 sets of retrospective data with 51 sets of recruited data from the metabolic syndrome study (n = 125) and analysed the accuracy of differential white blood cell counts performed with FCB-DM compared to acknowledged standard automated venous blood pathology counts.

3.1 Metabolic Syndrome Study

3.1.1 Target population, sample selection and size.

Data collection focussed on two population groups:

1. Obese participants with a waist measurement ≥ 88 cm for women and ≥ 102 cm for men (Obese)
2. Participants with a waist measurement < 80 cm for women and < 94 cm for men (Healthy)

The above mentioned thresholds, based on World Health Organisation guidelines (World Health Organisation, 2011), were used to determine participants eligibility in the initial telephone screening, based on the subjects' responses.

After consultation with the University's research methodologist, it was agreed that 70 would be the minimum number of participants to give statistically significant results and allow completion of the study within the two-year time frame.

3.1.2 Participant recruitment.

Permission was obtained from the Director of Coorparoo Integrated Health Care Centre (CIHCC), Brisbane, for information letters to be sent out to potential participants in the clinic database and Health World sales representatives in Brisbane and Lismore/ Northern Rivers were contacted and supplied information letters for prospective participants (Appendix G). A university-wide email was sent out to SCU staff and students in Lismore, calling for interested participants (Appendix H).

The student researcher initially screened respondents over the telephone or via email to assess their suitability. The screening process included gathering information about participant's waist measurement, age, medications and health to fulfil the inclusion and exclusion criteria listed in 3.1.3. The details of the involvement required were explained to eligible participants, and, if they agreed to be involved, they were sent an information sheet and consent form. After screening for suitability, each eligible participant was allocated a number (1 – 100 for Brisbane and 101 – 200 for Lismore), which was used for identification of the data. Research assistants then contacted the participants to organise appointments for FCB-DM and venous blood pathology testing.

3.1.3 Inclusion and exclusion criteria.

As part of the initial telephone screening, the following exclusion criteria applied:

- People under 21 years of age
- Pregnant or breast feeding women
- People on the following medications
    - Anti-inflammatories such as:
        - Glucocorticoids
        - Non steroidal anti-inflammatories (NSAID's)
        - COX 2 inhibitors, methotrexate, and quinine as these can decrease ESR; anti-inflammatory supplements
    - Anti-lipidaemic medications and supplements such as:
        - HMG CoA reductase inhibitors or statins such as Lipitor (atorvastatin), Mevacor (lovastatin), Zocor, Simvastatin, Crestor

  - Bile acid sequestrants such as Cholestyramine (Questran), Colestipol hydrochloride (Colestid, Colesevelam)
  - Niacin in high doses as it increases activity of lipase, reduces the metabolism or catabolism of cholesterol and triglycerides and can cause hyperglycemia
  - Fibric acid derivatives that activate lipase that suppress the release of free fatty acid from adipose tissue, inhibit synthesis of triglycerides in the liver, and increase secretion of cholesterol in the bile
  - Cholesterol absorption inhibitors such as ezetimibe (Zetia)
- People on platelet aggregation inhibitors such as aspirin that inhibit cyclooxygenase
- People on warfarin and statins.

- Participants with clotting disorders such as thrombocytopenia, Von Willebrand's disease, hemophilia and thrombophilia
- Participants undergoing cancer treatment such as chemotherapy and/or radiation
- Participants with diabetes or a history of hypoglycemia

3.1.4 Study design.

Much of the research into metabolic syndrome utilises venous blood pathology testing as outlined in Table 2. We were interested to see if any of these tests correlated with variables observed in FCB-DM by specifically investigating fibrin, platelet and chylomicron remnant presence, and comparing and/or correlating them to the acknowledged standard set by venous pathology testing. Queensland Medical Laboratories (QML) carried out the venous blood pathology tests.

The FCB-DM and glucose data collection was carried out at two locations:

A. Brisbane – CIHCC, and
B. Lismore – The Lismore campus of School of Health and Human Sciences, Southern Cross University.

Participants were mailed an information sheet together with a consent form to sign and return in a pre-paid envelope prior to their involvement in the study. Participants visited a QML collection centre at 8:00 to 8.30 am and had fasting

venous blood sample taken by a trained phlebotomist. The venous blood pathology tests requested are outlined in Table 4.

Table 4. FCB-DM and venous pathology tests comparative variables.

Test name	Specific pathology	Medical indication	FCB-DM variable
Eosinophil Sedimentation Rate	ESR	Raised in inflammation	• Fibrin presence • Platelet aggregation
C-reactive protein	CRP	Raised in inflammation (CRP is an acute phase protein)	• Fibrin presence • Platelet aggregation
Fasting blood glucose	Blood glucose	Blood sugar regulation	• Chylomicron remnant presence/ clearance
Fasting insulin	Insulin	Insulin sensitivity	• Chylomicron remnant presence/ clearance
HbA1c	Glycosylated haemoglobin	Blood sugar regulation and Insulin sensitivity	• Fibrin presence • Chylomicron remnant presence/ clearance
Lipid studies	• Triglycerides • HDL • LDL	Lipid metabolism	• Chylomicron remnant presence/ clearance
Differential white blood cell count	Differential white blood cell count	Immune function Inflammation	• Differential white blood cell count

Participants then travelled straight from the QML collection centre to CIHCC or the University for fasting FCB-DM analysis and blood glucose measurement. Participants then had their height, waist and hip circumferences measured and were weighed for BMI calculation. These measurements took 20-30 minutes to complete.

The participants were then given a test meal, a smoothie with 165 mL of coconut milk, containing 4g of carbohydrate, 19g of fat, 20g of protein and 2g of fibre, a total of 1,225.9 J (293 calories). This was consumed within 15 minutes. Two hours after the test meal, blood glucose measurement and FCB-DM analysis was repeated. The participants were then discharged with a thankyou snack pack (e.g. a protein bar, donated by Health World Ltd., or raw nuts and a bottle of water purchased for the study), and given the contact details of the researcher in case any issues or questions arose.

Example of a timetable for the testing day:

- No later than 10 pm the night before the research day participants were asked to eat a meal consisting of protein and salad or vegetables with minimal carbohydrates (for example, steak and vegetables; chicken and salad; tofu stir fry with no rice) and not to consume alcohol. They may also have a cup of tea or coffee with or without sugar.
- 7.30 am and 8.30 am: Fasting venous blood taken at a QML clinic of participant's choice.
- 9:00 - 9.30 am: Fasting FCB-DM analysis, blood glucose, weight, height, waist and hip circumference measured.
- 9.30 - 9.45 am: A test meal was provided – protein smoothie.
- 11.45 - 12:00 am: FCB-DM and blood glucose tests repeated to observe changes in the blood variables.

3.1.5 Blinding.

All venous pathology results were held securely on the QML database and only released to the researcher when all data collection was completed.

3.1.5.1 Blinding – Brisbane.

A research assistant, a qualified and experienced FCB-DM practitioner, RR, was employed. Once participants were approved for the study RR organised appointment times with the participants who were allocated a random identification number between 001-100 to be used throughout the project. RR kept this list, together with participants' consent forms and anthropometric measurements, in a secure place in the clinic, inaccessible to the student researcher until all data was collected. This ensured the student researcher was blinded to the sample being analysed.

On the day of testing, the participants only had contact with RR who carried out the measurements and performed the finger prick blood collection for blood glucose measurement and prepared the slides for FCB-DM analysis. Immediately after preparation, these slides were delivered to the student researcher, who assessed the quality of the slide and performed the FCB-DM screening. RR then gave the participant the test meal to be consumed within 15 minutes and repeated the finger

prick blood collection two hours after the meal was finished.

3.1.5.2 Blinding – Lismore.

As the student researcher remained in Brisbane, blinding of the data collection in Lismore happened automatically. The assistant researcher, SK, a qualified and experienced FCB-DM practitioner, was able to liaise with the participants, book appointments, allocate participant numbers between 101 and 200, perform the finger prick and slide preparation and take photos of the FCB-DM analysis. SK then gave the participant the meal, instructed them to consume it within 15 minutes, and took the above-mentioned measurements before repeating the FCB-DM two hours later. The images were supplied to the student researcher in Brisbane for analysis with the code numbers only, and thus no contact was made between the Lismore participants and the student researcher beyond the initial screening phone call or email.

3.1.6 Blood collection procedure – pathology.

As stated previously, all pathology tests were performed by the Queensland Medical Laboratories (QML). Blood was collected by a trained phlebotomist and involved placing a sterile disposable double ended needle into a vein of the participant's arm and collecting 5 mL of blood. This needle remained in the arm for about 1 minute, as the necessary blood was collected into a serum separation tube (for blood glucose, cholesterol and triglycerides) and EDTA tube, lavender topped (full blood count, ESR, CRP) (Queensland Medical Laboratory, 2010).

Occasionally, when blood is extracted, patients experience minor bruising around the vein. A sterilised cotton ball and pressure was applied to the area for a period of 5 minutes after the blood was taken to lessen the possibility of bruising. The wound was covered with a band-aid and participants were provided with post blood collection care sheet to reduce any risk. The method of blood collection was carried out according to the 'Universal Precautions' guidelines set out in the NHMRC publication 'Infection Control in the Health Care Setting' (National Health and Medical Researtch Council, 2010a).

Blood was transported to the commercial pathology laboratory where blood

haematology, including the full blood count, leucocytes, erythrocytes and platelets were analysed on a fluorescent flow cytometry Symex XE-2100 automated analyser machine. Lipid and Iron studies were analysed on a Siemens labcell Advia 2400 machine.

3.1.7 Blood collection procedure - fresh capillary blood analysis.

An alcohol wipe was used to clean the third or fourth fingertip, then the lateral side of the finger was pierced with an Accu-Chek Softclix retractable, sterile lancet. The skin around the prick site was gently stretched to encourage a blood droplet to appear. The first drop was used for glucose measurements and wiped away with a Kimberly-Clarke professional Kimwipe to prevent appearance of any damaged cells from the wound. A clean Menzel-Gläser (Erie Scientific Company, Germany) 1 mm microscope slide was carefully touched to the second drop of blood without making contact with the skin. A Deckgläser (Erie Scientific Company, Germany) 22 x 40 mm cover slip was applied to the slide within 10 seconds of the drop of blood being collected. This was done gently and from as close to the slide as possible (no greater than 5 mm above) to avoid damaging the sample. A second slide was then prepared by wiping away any blood on the fingertip then stretching the skin around the prick site again. The research assistant collecting the sample wore Manufix sensitive powder free disposable examination gloves (B. Braun, Germany) that were disposed in a biological wastes container along with the used lancet needle immediately after use to avoid biohazard risk.

The blood sample should spread immediately and evenly over the slide and fill 2/3 of the coverslip with a discernible lysis zone in the centre. A timer was set from the moment the cover slip was in place. The analysis was recorded within 20 minutes of the sample being prepared as the progressive clotting process may obscure observations after 20 minutes (Metagenics, 2010).

The perimeter of the blood on the slide was exposed to the air, and quickly began to clot and was prone to damage and distortion. The central area of the slide is known as the ‘Lysis zone’ when the blood cells are damaged in the process of preparing the slide (Metagenics, 2010). Surface area tension between the slide and

coverslip caused the coverslip to become slightly concave, applying pressure to the blood sample and creating the lysis zone in the centre of the slide.

The area used for FCB-DM analysis is known as the ‘working zone’ of the slide. This zone is indicated below (Figure 12) and lies between the Lysis zone and the non-working zone.

Two slides were prepared as per the clinical application of the technique. This was to ensure there was a back up slide if the first was corrupted or damaged and was used as a reference when clinical observations were unusual and for quality assurance. In this research project only the first slide was used for analysis, as no such incident occurred.

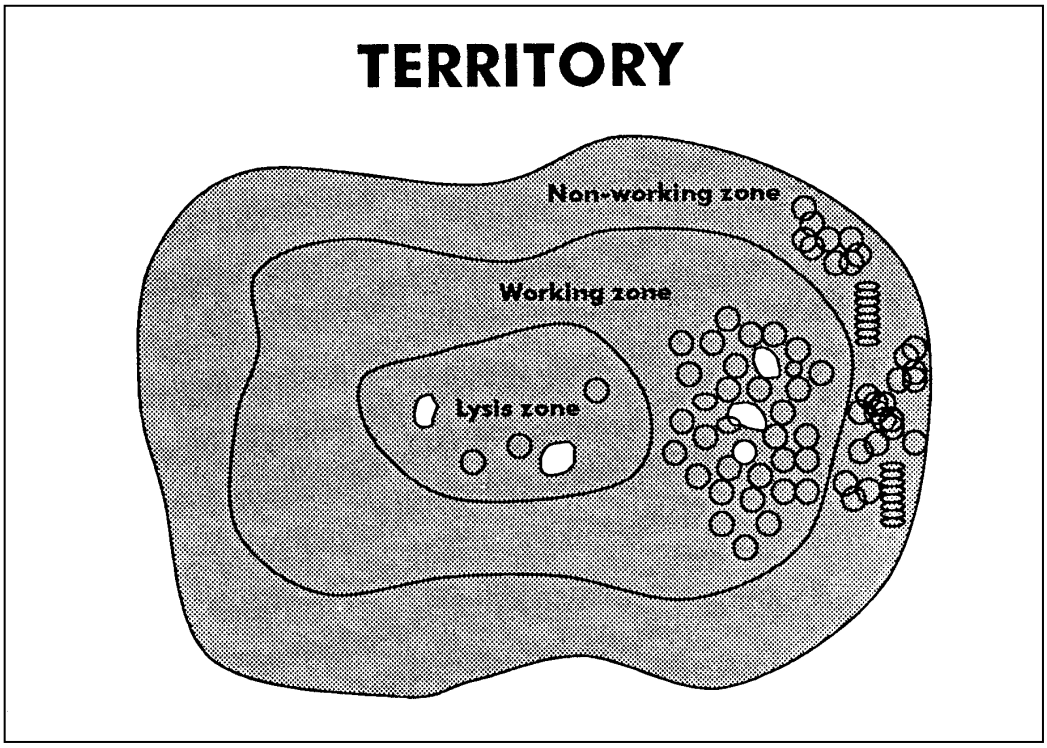


Figure 12. FCB-DM slide territories.
Health World Australia Ltd (Metagenics, 2010)

3.1.8 Fresh capillary blood analytical technique.

The slide was placed on the darkfield microscope stage and examined using the 10x objective to locate the working zone. One photo was taken at this magnification, and then the 40x objective was used to collect the images used for the

research. In Lismore, the slide was examined under a Prism Optical PRO 20/50 microscope (Prism Optical, China) with a darkfield cardioid oil immersion condenser using Plan acromatic objectives with an attached Watec 22IS camera (Watec, Japan). The Brisbane microscope was a Zeiss Axiolab drbKT fitted with a darkfield cardioid oil immersion condenser using Zeiss CP Achrostigmat objectives with an attached GOVideo CCS-C680HR digital camera (Sony, Japan).

Images of the blood cells were then captured with Metascreen™ software (Health World, Australia) using a camera attached to the trinocular tube at the eyepiece of the microscope. For each live blood screening, 15 consecutive fields of vision were photographed from a sample area of the slide, viewed at 400x magnification. The photographs were analysed once all the data was collected and measurements were recorded in an Excel spreadsheet.

The photographs of each field of vision were taken, beginning at the top left corner of the working zone and proceeding left to right, then down one field of vision, returning to the left and continuing (Figure 13) until 20 sequential fields were photographed.

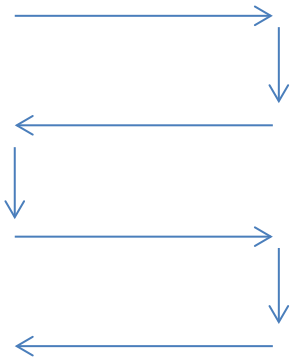


Figure 13. The direction of movement across the blood film.

A differential white blood cell count was carried out after the 15 photos were recorded and saved. The differential count involved counting 100 white blood cells as seen in sequential viewing fields, following the direction of movement as shown in Figure 13.

3.1.9 Microscope image calibration.

Calibration was necessary to translate the image size and focus to ImageJ software (Schneider, Rasband, & Eliceiri, 2012) for analysis.

- A slide micrometre with 0.01 mm intervals was placed on the darkfield microscope used in the data collection and photographed at 400x magnification.
- This image was opened using ImageJ software and, using the line-measuring tool. A straight line measuring 100 µm was selected and a scale set to the number of pixels per µm on the micrometre. The scale determined was 1.4 pixels to 1 µm for Brisbane setting and 4.4 pixels to 1 µm for Lismore setting.

3.1.10 Data collection.

For each participant –

- 15 images from the fasting sample and 15 images from the postprandial sample were captured in sequential viewing fields inside the working zone of each slide.
- Each image was analysed using ImageJ software –
    - The number of chylomicron remnants, visible as small white spherical particles approximately 1 µm were counted.
    - The number of platelets in each picture was counted. The average size of the platelets was obtained by measuring the area of each platelet using the drawing icon in ImageJ software to trace the external perimeter of each platelet in the image.
    - The number of fibrin clusters in each picture was counted. The average size of the fibrin clusters was obtained by measuring the area of each fibrin cluster using the drawing icon in ImageJ software to trace the external perimeter of each platelet in the image. The presence of any diffuse fibrin was graded 0-3 where 0 = not present, 1 = mild, 2 = moderate, 3 = severe.
- The average of the total of each variable's measurements were calculated for each participant's fasting and postprandial samples.

3.1.11 Statistical analysis.

Data was analysed using the Statistical Package of Social Science (IBM® SPSS® Statistics, Version 22). To assess normality of distribution in a small sample

size, two useful variables are the z-scores for skewness and kurtosis. Through SPSS, the statistical values of skewness (S) and kurtosis (K) were produced as well as their standard error (SE). The z-scores were subsequently calculated by subtracting the mean of the distribution (= 0) from the skewness/kurtosis value and then dividing it by the standard error of the distribution. If the z-score for skewness and kurtosis was between +/- 3.29 the variable was believed to follow a normal distribution (Field, 2009, pp. 139-140).

Alpha level was set at $p = .05$ as statistically significant. As per Pallant (2007, p. 145) correlation coefficients are regarded as strong if >.5, moderate if .3-.49 and weak if < .29. The relationship between FCB-DM findings and venous pathology test results were assessed with correlation coefficients as outlined in Table 4. Correlations were made using a bivariate correlation test and the strength of the effect size interpreted using Cohen's guidelines (Pallant, 2007).

The following statistical analyses were applied to the data to test the null hypotheses:

A. *The platelet area and number measured in fasting FCB-DM would not correlate to the platelet counts in fasting venous blood samples by conventional pathology tests.* Pearson's correlation coefficient or Spearman rank correlation were applied to the data to examine the relationships between FCB-DM platelet measures and venous pathology platelet counts.

B. *The presence of fibrin measured in fasting FCB-DM would not correlate to ESR and CRP in fasting venous blood samples by conventional pathology tests.* To examine the relationship between venous pathology measures of inflammation (ESR and CRP) Pearson's correlation coefficient or Spearman rank correlation were applied to the data.

C. *The presence of chylomicron remnants in fasting FCB-DM would not correlate to the lipid profile, glucose, insulin and HbA1c in fasting venous blood samples measured by conventional pathology tests; and there would be no difference in chylomicron remnant counts between the fasting and*

postprandial samples as measured by FCB-DM; and there would be no difference in fasting and postprandial chylomicron remnant counts between the normal and high BMI groups. Pearson's correlation coefficient (normally distributed data) or Spearman rank correlation (not normally distributed data) were applied to the data to examine the relationships between data. The fasting and postprandial chylomicron remnant counts were compared through paired sample *t*-tests.

3.1.12 Intra-measurer reliability.

To determine the intra-measurer reliability of the analysis, as part of the validation process, a third party person randomly selected 20 images from the data to be used in a reliability study. These images were renamed to ensure blinding, and then given to the principal researcher to analyse following the same methodology as outlined in 3.1.10. This data was compared to the original analysis of these images in a paired sample *t*-test, and Pearsons correlations. Technical error of measurement (TEM) was calculated following Kevin Norton's and Tim Olds methodology (Norton & Olds, 2000; Perini, Oliveira, Ornellas, & Oliveira, 2005).

3.1.13 Limitations and delimitations.

The nature of the FCB-DM technique is always going to rely on appropriate slide preparation and precise location of the working zone on the slide for correct and accurate information extraction. To minimise the potential errors in sample preparation we used only two experienced practitioners to collect the capillary blood and prepare the slides, and all the slides (Brisbane) and photographs (Lismore) were analysed by the student researcher to assess quality control, to ensure the slides were of a high standard. If the initial set of slides were not of a suitable standard, a second set of samples would have been prepared. Difference in the scale of the images between Brisbane and Lismore meant the data for the different collection centres could not be combined.

3.1.14 Ethics approval.

The ethical and legal responsibilities that researchers have towards participants in research reflect the basic ethical values of integrity, respect for persons,

beneficence and justice (National Health and Medical Researtch Council, 2009, 2010b). Ethics approval for this research project was granted on the 7th of May 2013, by the Southern Cross University Human Research Ethics Committee reference number ECN-13-111.

3.2 Differential White Blood Cell Study

3.2.1 Study design.

The data were collected from two separate sources. The first was from a retrospective group (Retrospective) whose data was collected from files at a naturopathic clinic. The collection process involved systematic selection from the 3000 archived clinic files obtained during the period from 2008 to 2012.

A total of 172 patients who satisfied the criteria, as outlined in 3.2.2 were contacted in writing, and consent was obtained from 72 eligible participants. Two participants contributed two separate sets of FCB-DM and venous blood pathology data for the study obtained at different time points. The automated blood pathology analysis had been carried out at a number of different commercial blood pathology laboratories, namely QML Pathology Service and Sullivan Nicolaides Pathology.

The second data set was collected as part of the Metabolic Syndrome study carried out at the same clinic during 2013 (Recent). As outlined in section 3.1.4, participants' (n = 51) fasting venous blood samples were taken and automated differential white blood cell counts were performed at a commercial pathology laboratory (QML Pathology Service), then fasting fingerpick blood samples were obtained within one hour of venous sampling for FCB-DM differential white blood cell counts.

3.2.2 Inclusion and exclusion criteria.

The retrospective data selection was based on patients' suitability for this study defined by the following inclusion and exclusion criteria:

- Each of the patients should have had a complete FCB-DM screening performed by the same FCB-DM practitioner;

- Data should be collected from files of any patient who had an automated differential white cell count and a FCB-DM differential white cell count completed within 14 days of each other; and
- Patients who were, at the time of screening, under 18 years of age, pregnant, undergoing chemotherapy, or who were on immunosuppressive agents, non-steroidal anti-inflammatory drugs or antibiotics should be excluded.

The inclusion and exclusion criteria for the recent data are outlined in section 3.1.3.

3.2.3 Blinding.

The researcher who performed FCB-DM was blinded from the automated venous blood pathology results until the data collection was completed.

3.2.4 Procedures.

The Hemaview^{TM} FCB-DM technique, as used in analyses for both the Retrospective and Recent groups, is outlined in section 3.1.8. For every participant the FCB-DM differential cell count was recorded for analysis. One hundred leukocytes as seen in sequential viewing fields were identified and counted. Cells observed were neutrophils, lymphocytes, monocytes, eosinophils and basophils. As utilised in clinical practice, all the white blood cell counts analysed, either FCB-DM or automated venous blood pathology, were calculated as percentages of the total white blood cell count.

3.2.5 Data analysis.

Data was analysed using the Statistical Package of Social Science (IBM® SPSS® Statistics, Version 22).

The automated venous blood pathology and FCB-DM mean scores were calculated and an independent sample *t*-test was used to investigate the differences between the two white blood cell count techniques. To determine the relationship between the automated venous blood pathology and FCB-DM cell counts, analyses of correlation and covariance were carried out between each of the white blood cell types (neutrophils, lymphocytes, monocytes, eosinophils and basophils). Pearson's

correlation was performed for normally distributed data (neutrophil and lymphocyte) and Spearman's correlation was used for data not normally distributed (monocyte, eosinophil and basophil). A linear regression uses the presence of a linear relationship to predict the value of a dependent variable based on the value of an independent variable (Lund & Lund, 2013). In this case, the linear regression model was used to test if FCB-DM differential white blood cell counts could predict the automated venous pathology results.

3.2.6 Ethics approval.

Ethics approval was granted on Jan 10th 2012 (approval number ECN-12-002) for the retrospective data collection and 7th of May 2013 (ECN-13-111) for the recent collection by the Southern Cross University Human Research Ethics Committee.

4 Results

4.1 Metabolic Syndrome Study

4.1.1 Characteristics of the participants.

A total of 92 eligible participants were recruited (64 in Brisbane, 28 in Lismore). Ten of these eligible participants did not attend the agreed upon appointment time, five could not schedule appointments in the allocated timeframe, two were unable to participate due to illness and five chose not to participate. Thus, 70 sets of data were collected, 51 from Brisbane and 19 from Lismore, that were analysed and included in the statistical analyses described below. The Brisbane cohort included 41 women and 10 men, the Lismore cohort contained 14 women and 5 men. As per the WHO criteria for BMI (Table 3) the Brisbane cohort had 24 'healthy', 4 'pre obese' and 23 'obese ' participants and the Lismore cohort was comprised of 10 'healthy', 4 'pre obese' and 5 'obese' participants. The general characteristics of the participants are presented in Table 5, and the raw data is presented in Appendix I and J, and correlation results are tabulated in Appendix L.

Table 5. Participants' general characteristics mean values.

	Brisbane			Lismore		
	Number	Mean	Standard deviation	Number	Mean	Standard deviation
Age	51	45.1	14.5	19	45.7	12.2
Weight (kg)	51	81.2	25.4	19	78.1	20.2
Height (cm)	51	165.7	10.9	19	169	8.6
Waist (cm)	51	94.4	19.8	19	90.4	16.4
BMI	51	29.7	9.3	19	27.5	7.8

4.1.2 Data collection site differences.

A difference in the focal length or resolution of the cameras used at the two sites was discovered, in that when a micrometre ruler was used to correlate pixels to micrometres in Lismore, the ratio was 1 pixel = 4.4 µm, whereas in Brisbane the ratio

was 1 pixel = 1.4 µm. The difference in the results (Appendix I & J) was clearly evident and thus prevented the data being collapsed into one cohort, so the Lismore and Brisbane data sets were analysed separately.

4.1.3 Platelets – Brisbane.

The Brisbane and Lismore mean values of the FCB-DM and venous blood pathology platelet measures are presented in Table 6. The fasting and postprandial average platelet number and the fasting and postprandial average platelet area determined by FCB-DM were correlated with each other to check repeatability of the measurements. A moderately significant Spearman's correlation was observed between FCB-DM fasting average platelet area and FCB-DM postprandial average platelet area (r_s (51) = .37, p < .05).

Table 6. Participants' platelets mean values.

	Brisbane			Lismore		
	Number	Mean	Standard deviation	Number	Mean	Standard deviation
Venous Blood Pathology Platelets	50	239.9	45.5	19	249.3	77.8
FCB-DM Fasting Platelet Area	51	296.4	126.8	19	62.6	63.5
FCB-DM Fasting Platelet Number	51	5.6	2.5	19	5.3	2.8

Significant Spearman's correlations were observed between FCB-DM fasting average platelet area and venous blood pathology platelets (r_s (50) = .36, p < .05) and between FCB-DM fasting average platelet area and venous blood pathology CRP (r (50) = .44, p < .05).

A significant Pearson's correlation was also observed between venous blood pathology platelets and venous blood pathology CRP (r (49) = .48, p < .05). No correlations were found between the fasting average platelet numbers and venous blood pathology platelets (r (50) = .03, p < .831) or venous blood pathology ESR (r (50) = .13, p < .37).

4.1.4 Platelets – Lismore.

No correlations were found between the fasting FCB-DM average platelet area and fasting average platelet numbers (r (19) = .25, p < .3). Neither of the fasting platelet measures correlated with the venous blood pathology platelet counts (r (19) = .11, p < .65; r (19) = .03, p < .91) nor with ESR (r (19) = .26, p < .29; r (19) = .01, p < .96) or CRP (r (19) = .14, p < .56; r (19) = .06, p < .81).

4.1.5 Fibrin – Brisbane.

The Brisbane and Lismore mean values of the FCB-DM fibrin and venous blood pathology CRP and ESR measures are presented in Table 7. The fasting and postprandial fibrin numbers and the fasting and postprandial fibrin areas determined by FCB-DM were all found to correlate with each other. Strong significant Pearson's correlations were found between fasting FCB-DM fibrin number and fasting FCB-DM fibrin area (r (51) = .64, p < .05). Significant Pearson's correlations were also observed between fasting FCB-DM fibrin area and postprandial FCB-DM fibrin number (r (51) = .45, p < .05). Significant Spearman's correlation was found between postprandial FCB-DM fibrin area and fasting FCB-DM fibrin number (r_s (51) = .38, p < .05), fasting FCB-DM fibrin area (r_s (51) = .30, p < .05) and postprandial FCB-DM fibrin number (r_s (51) = .47, p < .05).

Table 7. Participants' FCB-DM fibrin and markers of inflammation mean values.

	Brisbane			Lismore		
	Number	Mean	Standard deviation	Number	Mean	Standard deviation
Venous Blood Pathology ESR	2	11.1	7.4	19	10.6	8.7
Venous Blood Pathology CRP	50	4	3.3	19	4.5	6.1
FCB-DM Fasting Fibrin Area	51	350.5	657.1	19	3.6	11.1
FCB-DM Fasting Fibrin Number	51	0.7	0.7	19	0	0.1
FCB-DM Fasting Diffuse Fibrin	51	0.4	0.5	19	0.1	0.6

Significant Pearson's correlations were observed between venous blood

pathology ESR and fasting FCB-DM fibrin number (r (51) = .69, p < .05) and fasting FCB-DM fibrin area (r (51) = .49, p < .05).

BMI was also found to correlate with ESR (r (51) = .29, p < .05), CRP (r (50) = .48, p < .05), fasting FCB-DM diffuse fibrin (r (51) = .56, p < .05) and postprandial FCB-DM diffuse fibrin (r (51) = .44, p < .05).

4.1.6 Fibrin – Lismore.

A significant, positive and strong Spearman's correlation was found between FCB-DM measures of fasting fibrin area and fasting fibrin number (r (19) = .99, p < .05).

A correlation was observed between fasting FCB-DM fibrin area and ESR (r (19) = .47, p < .05), but no correlation was seen between fasting fibrin number and ESR (r (19) = 0.42, p > .05). No correlations between the fasting FCB-DM fibrin measures and CRP were found (r (19) = .11, p > .05; r (19) = .09, p > .05).

4.1.7 Chylomicron remnants – Brisbane.

The Brisbane and Lismore mean values of the FCB-DM chylomicron and relevant venous blood pathology measures are presented in Table 8. There was a statistically significant difference between fasting and postprandial chylomicron remnant counts in the FCB-DM scores (t (50) = -10.10, p < .05).

4.1.7.1 Correlations.

The fasting chylomicron remnant FCB-DM results were positively skewed so the Log-transformed data were used for determination of correlation coefficients.

A significant but moderate Spearman's correlation was found between (transformed) FCB-DM fasting remnant counts and venous blood pathology triglycerides (r_s (50) = .30, p < .05). Significant Pearson's correlation between (transformed) FCB-DM fasting chylomicron remnant counts and $HOMA_B$ (r (47) = .31, p < .05) and $HOMA_{IR}$ (r (46) = .31, p < .05) was observed.

Significant Pearson's correlation between (transformed) FCB-DM fasting

chylomicron remnant counts and venous blood pathology fasting insulin (r (50) = .33, $p < .05$) and (transformed) FCB-DM postprandial chylomicron remnant counts and HbA1c (r_s (51) = .35, $p < .05$).

Table 8. Participants' FCB-DM chylomicron and lipid measures mean values.

	Brisbane			Lismore		
	Number	Mean	Standard deviation	Number	Mean	Standard deviation
FCB-DM Fasting Chylomicrons	51	21	21.3	19	24.4	32.6
FCB-DM Postprandial Chylomicrons	51	80.5	38.1	19	68.1	30.1
Venous Blood Pathology HbA1c	51	5.4	0.3	19	5.4	0.2
$HOMA_B$	47	127.2	52.6	19	130.1	53.2
$HOMA_{IR}$	46	1.6	1.1	19	1.4	0.9
Venous Blood Pathology Triglycerides	50	1.1	0.9	19	0.8	0.4

4.1.7.2 Influence of BMI.

The cohort was divided into two groups by BMI, healthy (BMI < 25) and obese (BMI ≥30). Three participants were excluded as they were in the "pre-obese" category (< 25 BMI < 30).

The mean of fasting actual chylomicron remnant number was 8.65 (transformed .94) for healthy and 16.14 (1.21) for the overweight groups respectively. Postprandial actual chylomicron remnant mean was 78.70 (1.90) for the healthy and 64.7 (1.81) for the overweight groups (Figure 14). Significant differences between the fasting and postprandial chylomicron remnant counts were found in both the healthy (t (22) = - 8.72, $p < .001$) and the obese groups (t (24) = - 6.12, $p < .001$).

The two tailed, paired sample t-test comparing transformed FCB-DM fasting and postprandial chylomicron remnant between the healthy and obese groups were not significant in either fasting (t (46) = - 1.79, $p > .05$) or postprandial (t (46) = - 1.24, $p > .05$).

4.1.8 Chylomicron remnants – Lismore.

The paired sample t-test for fasting and postprandial chylomicron remnant

counts was significant (t (19) = 7.72, p < .05). No significant correlations were found between the FCB-DM measures of fasting and postprandial total average chylomicron remnants with venous pathology triglycerides. The data was unable to be split into BMI group due to the small number of samples.

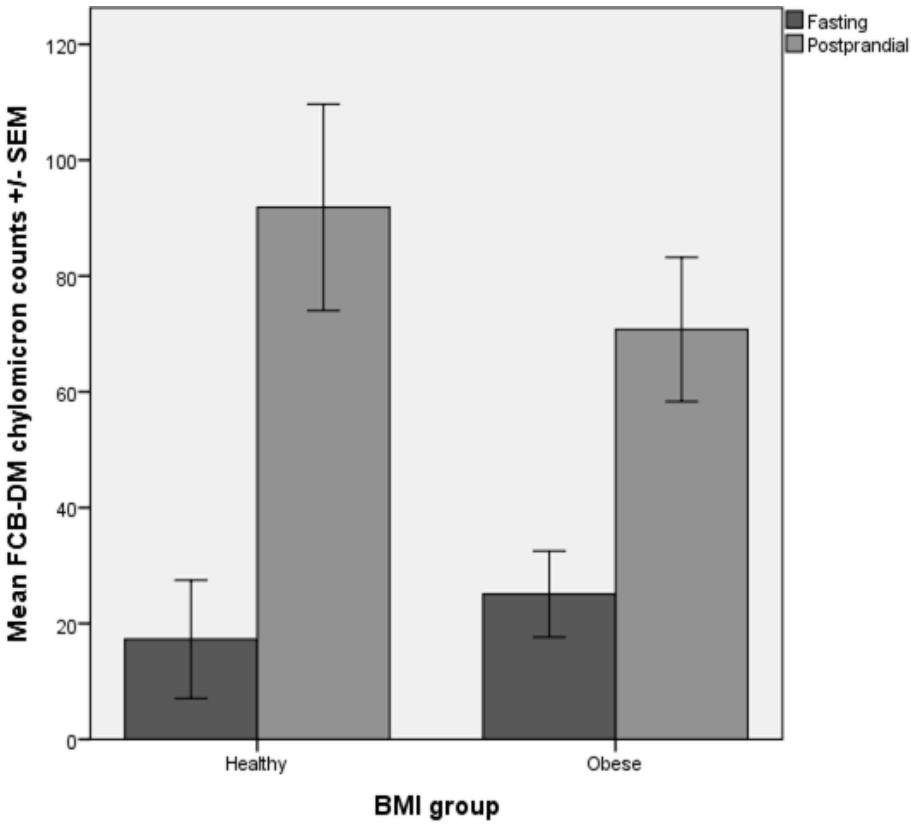


Figure 14. Chylomicron remnants and BMI.
Actual FCB-DM fasting and postprandial chylomicron remnant counts - healthy and obese.

4.1.9 Reliability of analysis.

Twenty images were randomly selected by a third party and analysed by the primary researcher following the method outlined in 3.1.11. Blinded, these results were compared to the results of the original analysis of these images. The paired sample t-tests for all FCB-DM variables were not significantly different i.e. platelet counts (t (20) = .57, p > .05), platelet area (t (20) = .59, p > .05), fibrin area (t (20) = 1.186, p > .05), and chylomicron remnant count (t (20) = .96, p > .05). Strong Pearson correlations were found between platelet number (r (20) = 1.0, p < .05), platelet area (r (20) = 1.0, p < .05), fibrin number (r (20) = 1.0, p < .05), fibrin area (r (20) = 1.0, p < .05) and chylomicron remnant number (r (20) = 1.0, p < .05). The relative technical

error of mean (TEM) was calculated to obtain the error expressed as percentage corresponding to the total average of the variable to be analysed. Relative TEM was 0% for fibrin number, 1.4% for fibrin area, 2.8% for platelet count, .11% for platelet area and 0.64% for chylomicron remnant count.

4.2 Differential White Blood Cell Counts

As shown in Table 9, here were significant differences between the two techniques in the mean scores of the total collapsed data set with significantly higher numbers in FCB-DM determined neutrophils (t (124) = -9.24, $p < .001$) than that in the venous blood pathology test. The FCB-DM lymphocyte and basophil counts were significantly lower than that of the automated venous blood pathology (t (124) = 8.56, $p < .001$; t (124) = 7.87, $p < .001$), respectively. No statistically significant difference was found between the FCB-DM and automated venous blood pathology tests in monocyte or eosinophil (t (124) = 1.25, $p > .05$; t (124) = 1.27, $p > .05$) counts, respectively.

Table 9. Differential white blood cell counts.
Combined retrospective and recent recruited mean percentage white cell counts (standard deviation) paired sample *t*-tests and correlation coefficient.

Full data Collapsed						
	Haematology mean %	Hemaview mean %	*t*	*p*	Correlation	*p*
Neutrophil	57.7 (8.9)	64.7 (9.9)	-9.24	0.001	*r* = .604	0.01
Lymphocyte	30.7 (9.1)	24.7 (9.0)	8.56	0.001	*r* = .627	0.01
Monocyte	8.8 (3.2)	8.3 (3.7)	1.25	0.214	*rs* = .32	0.01
Eosinophil	2.5 (1.8)	2.3 (1.9)	1.27	0.206	*rs* = .596	0.01
Basophil	0.5 (0.5)	0.1 (0.3)	7.87	0.001	*rs* = .132	0.14

4.2.1 Correlation.

There was a significant, positive and strong Pearson's correlation coefficient between the automated venous blood pathology and the FCB-DM counts for neutrophil (r (125) = .60, $p < .001$) and lymphocyte (r (125) = .63, $p < .001$) (both two-tailed) populations. There was also a significant and positive Spearman's correlation between the automated venous blood pathology and the FCB-DM counts

for monocyte (r_s (125) = .32, p < .001) and eosinophil (r_s (125) =.60, p < .001) (both two-tailed) populations. A significant Spearman's correlation coefficient was not found between the automated venous blood pathology and FCB-DM counts for basophil (r_s (125) = .13, p > .05, two-tailed) (Table 9, Figure 15).

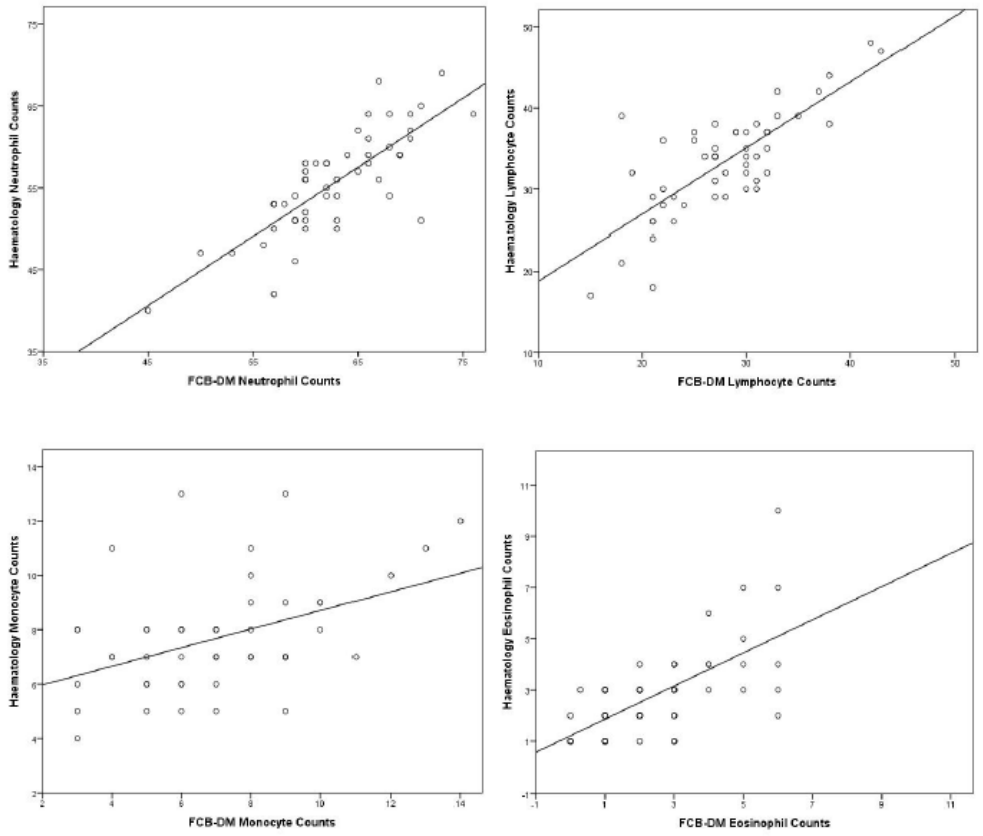


Figure 15. Differential white blood cell scatter plots.
Correlations between automated venous blood pathology and FCB-DM neutrophil, lymphocyte, monocyte and eosinophil counts.

5 Discussion

The major findings of the studies included significant correlations between:

- FCB-DM fasting average platelet area and venous pathology platelet counts and CRP.
- FCB-DM fasting fibrin number and fibrin area and venous pathology ESR.
- FCB-DM fasting and postprandial diffuse fibrin and BMI.
- FCB-DM fasting chylomicron remnant counts and venous blood pathology triglycerides and insulin.
- FCB-DM fasting chylomicron remnant counts and $HOMA_B$ and $HOMA_{IR}$ calculations.
- FCB-DM postprandial chylomicron remnant counts and HbA1c.
- FCB-DM neutrophil, lymphocyte, monocyte and eosinophil populations and venous pathology counts, and
- Significant difference between FCB-DM fasting and postprandial chylomicron counts.

5.1 Metabolic Syndrome Study

The aim of the study was to determine the validity of FCB-DM by examining the concurrent correlation between FCB-DM variables and venous blood pathology tests when screening for metabolic syndrome risk factors. The relationship between fasting FCB-DM variables of fibrin, platelets and chylomicron remnants, and fasting venous blood haematology measures were examined. In addition, the relationship between fasting and postprandial FCB-DM chylomicron remnants was examined. As stated earlier, a significant correlation assumes the relationship between variables is meaningful, but in reality may lack clinical relevance, thus a degree of discernment in interpretation is required (Salkind, 2012, p. 125). In relation to FCB-DM, a statistically significant relationship in these initial investigations of the technique indicates the potential for clinical validity. This does not necessarily equate to accuracy, but the correlations indicate a significant relationship that warrants further investigation.

5.1.1 Platelets.

No significant correlations were observed between the Lismore FCB-DM fasting platelet number and area with venous blood pathology platelet counts, most probably caused by an insufficient statistical power from the small number in the Lismore cohort.

Brisbane data demonstrated correlations between FCB-DM fasting and postprandial platelet area and fasting and postprandial platelet number, indicating platelet area presence in FCB-DM did not change from the fasting state to the two hour postprandial state.

Correlations were seen between the Brisbane FCB-DM fasting platelet area measures and venous blood pathology platelet count measures. These correlations were not observed with the FCB-DM fasting platelet numbers, possibly because of the small area of blood sample analysed in this study. Venous blood pathology guidelines for normal platelet counts range from 150 to 450 x 10^9/L (Queensland Medical Laboratory, 2013) and in this study participant platelet number results ranged from 2.2 to 9.8 platelets and area 186 to 358 x 10^9/L.

Bath et al.(1996) state that the peripheral platelet count is not a useful measure for information about platelet-related haemostatic function, unless the count is very low, whereas automated haematological analysis of mean platelet volume (MPV) is considered a more accurate measure of platelet activation (Coban, Ozdogan, Yazicioglu, & Akcit, 2005) and metabolic and cardiovascular risk (Chu et al., 2010; Coban et al., 2005). The FCB-DM platelet average area may well be an indicator of MPV, and warrants further investigation with a larger data set, to examine possible clinical applications.

Venous blood specimens are collected in EDTA tubes treated with anticoagulants. Research has shown that platelets swell on exposure to EDTA and MPV rises over time, most markedly in the first hour and a half thus timing of the testing is critical for accurate outcomes (Bath & Butterworth, 1996; Giles, 1981). No anticoagulants are used in the FCB-DM process, so distortions of molecular features

are minimised (Slayter, 1989)

C-reactive protein correlated with both venous blood pathology platelet counts and fasting FCB-DM platelet area. CRP is an immunoglobulin that initiates the inflammatory response in reaction to increasing IL-6 secretion from macrophages and T-cells (Hoffbrand, 2006). CRP rapidly increases in the body, within hours, in response to a stimulus. Elevated CRP levels are commonly observed in patients with obesity and metabolic syndrome (K. G. M. M. Alberti et al., 2006; Haffner, 2006; Saltiel & Kahn, 2001; Siegel & Ermilov, 2011; Visser et al., 1999; Yudkin, 2003). Obesity and visceral adiposity have been shown to correlate with CRP levels in a number of studies (Lemieux et al., 2001; Mendall et al., 1997) echoed by the correlations observed in this study between BMI and both CRP and ESR. Elevated CRP levels have also been observed in patients with high triglycerides and low HDL, and fasting insulin levels (Hak et al., 1999; Mendall et al., 1997; Yudkin, Stehouwer, Emeis, & Coppack, 1999).

Abdominal obesity is independently associated with an elevated risk for atherosclerosis and cardiovascular disease (Anfossi et al., 2009; Coban et al., 2005; Perez-Campos-Mayoral et al., 2014). Dyslipidaemia and endothelial dysfunction trigger platelet hyperactivity, further increasing the risk of thrombotic arterial and venous events (Hyson, Paglieroni, Wun, & Rutledge, 2002; Mertens & Gaal, 2002; Nieuwdorp, Stroes, Meijers, & Buller, 2005). For this reason, it is important to have effective methods for identifying this hyperactive platelet state. FCB-DM fasting platelet area may be a useful clinical indicator of CRP concentrations seen in acute inflammation.

5.1.2 Fibrin.

Correlations were observed between FCB-DM fasting and postprandial fibrin area and fasting and postprandial fibrin number in the Brisbane data, but only correlations between fasting FCB-DM fibrin number and area correlated in the Lismore data. This is most likely due to the small sample size and therefore poor statistical power. Nevertheless these correlations indicate little change to the fibrin cluster area and number concentration from fasting to postprandial in FCB-DM samples.

Interestingly, no correlations were seen between diffuse fibrin in the fasting and postprandial measures in either the Lismore or Brisbane groups. One explanation for this could be that the postprandial state is more inflammatory than the fasting. Postprandial release of glucose and fatty acids potentially outstrip the capacity of oxidative phosphorylation leading to an increase in free radicals (O'Keefe, Gheewala, & O'Keefe, 2008). This postprandial oxidative stress triggers atherogenic changes including dyslipidaemia, platelet activation and impaired fibrinolysis, in the patient with metabolic syndrome or diabetes suffering from chronic hyperglycaemia (L. Monnier et al., 2006; O'Keefe et al., 2008). Diffuse fibrin presence as seen with FCB-DM may have the potential to be a more sensitive marker of postprandial inflammation than fibrin cluster number and area. This is supported by the correlations observed in this study between BMI and both fasting and postprandial FCB-DM diffuse fibrin, and correlations of ESR and CRP with postprandial diffuse fibrin.

The fasting and postprandial FCB-DM fibrin average area and number measures correlated with ESR. ESR is the rate at which erythrocytes sediment in one hour. It is a common test for non-specific inflammation in the body, used to measure the acute phase response. A raised ESR indicates increased concentration of fibrinogen and immunoglobulins in the blood (Kumar & Clarke, 2005, p. 421). The results of correlations in this study demonstrate this association between fibrin presence in the capillary blood sample and a patient's ESR in line with in a paper by Hung et al. who found that increased concentration of fibrinogen accelerates erythrocyte sedimentation (Hung, Collings, & Low, 1994).

5.1.3 Chylomicron remnants.

Significantly different FCB-DM measures between fasting and postprandial chylomicron remnants were seen in both the Lismore and Brisbane groups. This indicates the chylomicron remnant concentration, observed in FCB-DM, changed from fasting to postprandial, an expected result in light of the literature discussed in 2.8.3. Interestingly, when separated by BMI, no statistically significant difference was observed between the healthy and obese groups in the fasting chylomicron remnant

counts, nor in the postprandial chylomicron remnant counts. As shown in Figure 14, the healthy group had non-statistically significant lower fasting chylomicron remnants than the obese group, but higher in the two hour postprandial counts. The timing of the presence of chylomicron remnants in the blood is dependent on digestion, absorption and metabolism of lipids. The literature agrees that postprandial hyperlipidaemia occurs in healthy people, but is more persistent in the obese and metabolic syndrome patients (Cassader et al., 2001; Cohen, Noakes, & Benade, 1989; Cohn, 2006; Duttaroy, 2005). Thus a two hour postprandial chylomicron remnant measure may be capturing the peak concentration in the healthy participants, but a concentration still on the rise, or fall, in obese participants. Or the statistical power of the data may not be strong enough to show a correlation with only 23 healthy and 24 obese sets of data. Besides being a disease in its own right, obesity is a risk factor for many other disease states (Formiguera & Canton, 2004). The complexity of obesity and abdominal adiposity, and the impact on metabolism, inflammation, liver function, hyperlipidaemia and endocrine function may well be clouding the data. For example decreased liver function or excessive fat consumption will result in an increase in the number, or a delayed clearance, of chylomicrons (Vitetta et al., 2005).

In the Brisbane data, fasting chylomicron remnants correlated with venous blood pathology triglycerides, that is, the higher the participant's triglyceride measures, the higher the fasting chylomicron remnant count in FCB-DM was. Correlations were also observed between fasting FCB-DM chylomicron remnant measures and venous blood pathology insulin, $HOMA_B$, $HOMA_{IR}$. As discussed earlier, triglyceride measures are used in clinical practice as a diagnostic indicator for poor lipid metabolism, atherosclerosis, cardiovascular disease risk and metabolic syndrome risk (Bansal et al., 2007). Patients with higher fasting insulin, higher HbA1c and raised triglycerides are considered at risk of metabolic syndrome (Balkau & Eschwege, 2005; Dominiczak, 2003a; Eckel et al., 2005; Ferrannini & Balkau, 2002; S. M. Grundy et al., 2004). Persistent chylomicron remnant presence observed in FCB-DM may be an indicator of obesity related hyperlipidaemia. $HOMA_B$ and $HOMA_{IR}$ calculations, based on a patients fasting glucose and fasting insulin measures, are used as indicators of beta cell function and insulin resistance respectively in clinical medicine and as an epidemiological tool (Wallace et al., 2004). Thus the observation of higher fasting FCB-DM chylomicron remnants potentially

indicates insulin resistance risk. These correlations indicate fasting FCB-DM may be a useful tool in monitoring cardiovascular disease and metabolic syndrome risk.

It is interesting to note that a correlation was observed between postprandial FCB-DM chylomicron remnants and HbA1c measured from fasting venous blood. HbA1c, determined by both fasting and postprandial glucose levels, is an indicator of a person's glycaemic exposure over the past 2-3 months (Woerle et al., 2006). Two cross-sectional studies suggest that reducing high HbA1c levels in patients is best achieved by targeting postprandial hyperglycaemia (Louis Monnier, Lapinski, & Colette, 2003; Woerle, Pimenta, Meyer, & et al., 2004).

Cooper et al., in a 12 hour study of 31 non insulin dependent diabetic and control subjects, found that chylomicron remnant clearance had a significant relationship with the release of insulin precursors (M. B. Cooper, Tan, Hales, & Betteridge, 1996). They concluded that 'abnormal postprandial lipidaemia in non insulin dependent diabetes is associated with beta-cell output, possibly mediated by the availability of free fatty acids' (M. B. Cooper et al., 1996). The correlation with FCB-DM postprandial chylomicrons is interesting, and further investigation into this link between postprandial hyperglycaemia, hyperlipidaemia and HbA1c in a larger cohort may shed light on the usefulness of FCB-DM as a clinical monitoring tool.

5.2 Differential White Blood Cell Study

The automated haematology and FCB-DM techniques analyse different volumes of blood from samples obtained from vein and capillary respectively, so variations in the test results would be expected (Kayiran et al., 2003; Lee & Tsai, 1989; Simmonds, Baskurt, Meiselman, & Marshall-Gradisnik, 2011). This is demonstrated by the significant difference in the mean scores between the two techniques in neutrophil, lymphocyte and basophil counts. The reference range for basophil counts in normal venous blood is ≤3%, therefore a statistically significant correlation is difficult to be observed in a sample size of 125. Also, there is contention in the literature in regard to the reliability of the testing of basophils with automated haematology machines (Amundsen, Henriksson, Holthe, & Urdal, 2012). The clinically valuable observation here is the significant correlation between the two

techniques, indicating they trend in parallel, however the test-retest reliability of the cell counts by FCB-DM, and the normal % ranges of cells, needs to be established.

There is conflicting information in the literature about blood constituent differences between venous and capillary blood. Numerous studies have shown that total leukocyte counts are higher in capillary blood than that in venous blood (L. N. Daae et al., 1988; Jouault et al., 1990; Schalk et al., 2007; Schalk et al., 2008; Yang et al., 2001) and these variations were greater among neutrophil populations and least among lymphocyte populations. Schalk et al. (2009) published a review of the literature investigating capillary and venous blood count parameters. The populations studied in these reports included children and adults, healthy and sick. They found that on average, the capillary white cell counts were above the corresponding venous values by 9.5% (1.2 x 10^3/ μL) (r = 0.98–1.0). Capillary neutrophil counts were on average 8.9% (0.68 x 10^3/ μL) (r = 0.81–0.98) greater than the corresponding venous counts, and greater in children (11%; 0.91 x 10^3/ μL) than in adults (4.7%; 0.22 x 10^3/ μL) (Schalk et al., 2009). In contrast, Dreyer et al. (Dreyer et al., 1994) analysed capillary and venous samples from 50 adults and found no difference in mean total white cell counts and the differential spread was concordant between the two samples. In two other studies with n=447 (Schalk et al., 2008) and n=50 (Lee & Tsai, 1989), pairs of capillary and venous blood samples from volunteers with a variety of health conditions were analysed using automated flow cytometry analysers. No statistically significant differences were seen in the absolute white cell and neutrophil counts in either of the studies.

The slightly higher average mean of neutrophil counts (p <.05) in FCB-DM (M = 64.70 ± 9.96) than in venous haematology (M = 57.74 ± 8.85) as observed in this study was in agreement with Schalk et al. (2009). The most significant difference in the white cell populations was with monocytes, a phenomenon that was also observed by Swaim (Swaim, 1991).

Both the Retrospective and Recent cohorts included participants of various ages (23 to 70 years) and health status, which ranged from healthy to participants with cardiovascular disease, obesity, metabolic disorders, and/or endocrine dysfunction.

Regardless of the cohort age, gender and health status variation, and despite the difference in the mean scores, significant correlations were observed between the two techniques in their neutrophil, lymphocyte, monocyte and eosinophil blood counts.

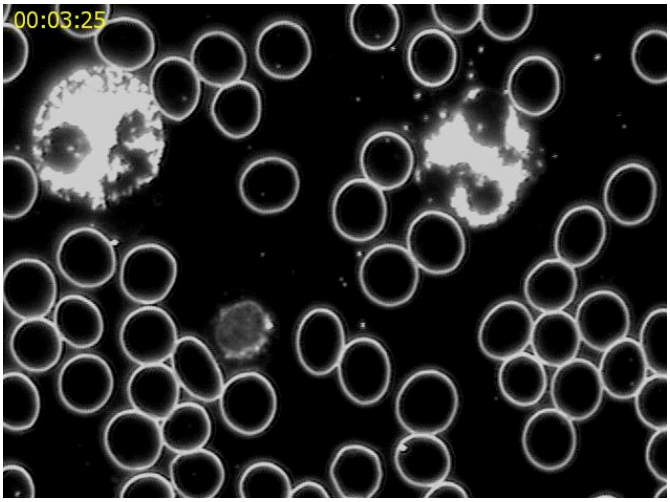
Cell counting with automated analysers is considered rapid, cost effective, efficient and statistically significant given approximately 8000 cells are counted (Kamath, 2011). Although the technology is advancing, the disadvantage of automated cell counts is that they offer limited morphological information (Ceelie, Dinkelaar, & van Gelder, 2007). Though manual blood smears are utilised by some pathology testing laboratories, they are not automatically included in the full blood count. An important advantage of the FCB-DM technique is the ability of the scientist and clinician to observe leukocyte morphology in a fresh, unstained, untreated blood sample. If further research can demonstrate acceptable validity and reliability, the FCB-DM technique poses a viable health-screening tool at point of care, for monitoring leucocyte morphology, counts and immune function.

Figure 16 (a) highlights an observation that became apparent during the data collection. The image contains two neutrophils, the large bright cells in the top right and left of the image. The neutrophil on the left of the image appears normal, with an intact outer membrane, visible granular particles and three dark nuclei. The neutrophil on the right is disrupted, the outer membrane of the cell is ruptured and the granular particles can be seen leaking into the blood sample around the top of the cell. These granular particles are similar in size and appearance to chylomicron remnants. These granular particles have the potential to influence and alter a chylomicron remnant count.

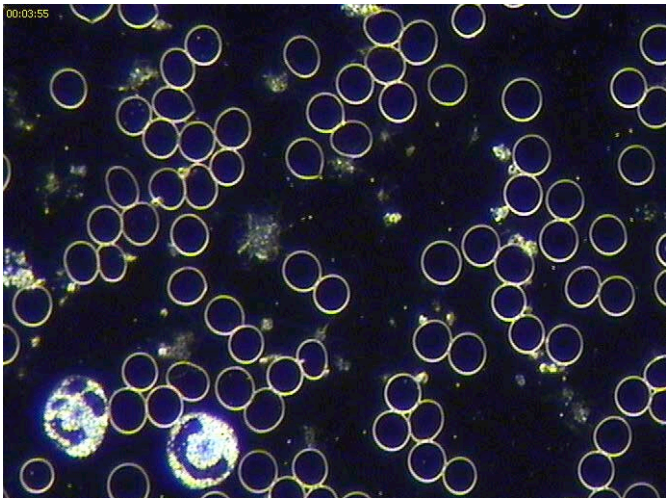
For every neutrophil in circulation, 50-100 mature cells are held in the bone marrow reserve (Zweifach, Grant, & McCluskey, 2014). With their short life span, this means a massive turnover and large numbers of cells lysing, passing out of the body through the intestine, skin and lungs or taken up by macrophages in tissue (Zweifach et al., 2014).

Studies of disrupted neutrophils reveal they lyse at a pH of 5 or lower and have phagocytic enzymes associated with, or contained within them (E. L. Cooper,

1983). These lysed neutrophils were only present in one participant's FCB-DM images and all care was taken to identify the critical areas and avoid any major alteration to the chylomicron remnant count. It should be noted that there will be a population of patients that would confound the chylomicron remnant count.



(a)



(b)

Figure16. Comparison of data images between Brisbane (a) and Lismore (b). Illustrating the difference in resolution of the images between Brisbane (a) 1 pixel = 1.4 µm and Lismore (b) 1 pixel = 4.4 µm. Two neutrophils are visible in both images: in image (a) the neutrophil on the left is normal, the neutrophil on the right is lysed; in image (b) both neutrophils in the bottom left are normal.

5.3 Methodological Limitations in the Metabolic Syndrome Study

The major strength of this study was that it endeavoured to provide preliminary evidence concerning the validity of a clinical screening tool for metabolic syndrome which, to date, there has been little scientific enquiry. A number of

limitations came to light during the roll out of the project, and are discussed here.

First the disparity of results between the testing sites (Brisbane and Lismore) reduced the usable sample size significantly, as the two sets of data could not be collapsed. The significant difference in the resolution of the images in ImageJ, as seen in Figure 16, had different pixel ratios. This could have been avoided by recruitment from only one location, but time limitations drove the decision to expand the recruitment to the two locations. This resulted in a smaller cohort from one location than anticipated and therefore the potential for less statistical power. The disparity in the pixel ratio did not affect the collection of the data for the differential white blood cell study, as the differential count was carried out at the time of the FCB-DM testing directly from the fresh blood sample.

Second, there were missing data caused by occasional omissions in taking blood samples and in completing specific tests by the collaborating venous pathology laboratory. As all the data collection occurred on one day, and timing in regard to fasting and postprandial blood samples was important, there was only a small window for error regarding venous blood collection issues. This further contributed to the shrinking of the sample size.

A third limitation of this study pertains to the measurement of platelet area. As can be seen in Figure 5, the circumference of the platelets can be difficult to define, obviously resulting in disparity in the area measure. Although the reliability of the testing showed little variation between the area measures, it is important to note that variation is probable.

The lack of prior research in the field and on the FCB-DM technique that might have been used as a guide in developing methodology presented its challenges to an early career researcher. Time restraints in the recruitment phase made it impossible to match the gender and age between the healthy and overweight groups, and thus the target groups included in the study were broad. The disadvantage of using a diverse sample is that the lack of homogeneity may introduce confounding factors that may affect the results and outcomes. Nevertheless, our data did successfully demonstrate significant and relevant correlations, in spite of the broad range of participant details, as well as the variable time period between the two blood

samplings, at least for the differential white cell measures, that help to justify the validity of FCB-DM compared with conventional venous blood analysis.

5.4 Methodological Limitations in the Differential White Blood Cell Study

A limitation in the study was the use of the retrospective data with up to 14 days elapsed between the two tests compared to the Recent group which had only one hour between the two tests. The dynamic nature of the immune system is such that changes in white blood cell populations occur in response to the body's requirements. White blood cell numbers would be expected, over time, to change with an individual's health status. Future research on the influence of time on the correlations between automated haematology and FCB-DM differential white blood cell testing would be of clinical value.

5.5 Future Study Directions

Future research should focus in two distinct directions, the determination and validation of specific variables in FCB-DM: what exactly we are observing and how do we know this, and only then can the validation of the technique and its use in clinical setting be researched.

As the observations of FCB-DM are largely subjective, further and thorough investigations of reliability and reproducibility are necessary to validate the technique. Small projects have been carried out in attempts to investigate inter practitioner reliability (Petric et al., 2010), but more scientifically rigorous and larger studies are required.

Larger, gender and age matched cohorts would improve the rigor of studies into FCB-DM validity in assessment of metabolic syndrome risk factors. More specifically targeted groups would help focus the results and outcomes, for instance the selection criteria targeting a more specific disease state than the complex and multifactorial metabolic syndrome.

Given the similarity of the techniques, an examination of FCB-DM compared

to blood film pathology, specifically around the manual differential white blood cell count and platelet number and area calculations, may shed more light on the accuracy of the technique.

Venous blood measures were only collected in the fasting state in this study due to financial and time restraints, but future comparisons of both fasting and postprandial FCB-DM and venous blood pathology measures would be of clinical importance.

Further research project areas of focus may include:

- A clotting speed study observing FCB-DM platelet and fibrin presence in patients on warfarin with matched controls.
- Chylomicron remnant comparison study on patients with fatty liver disease and matched controls.
- Chylomicron remnant comparison study on non insulin dependent diabetics and healthy matched controls.
- A time course study of postprandial venous blood pathology and FCB-DM parameters measured hourly after a test meal for 6 hours.
- Comparison of blood film manual counts and FCB-DM differential white blood cell counts.
- Comparison between differential white blood cell counts with FCB-DM and automated capillary counters differential white blood cell counts.
- Comparison of FCB-DM and automated pathology differential counts on blood taken simultaneously.

6 Conclusions

This research provides new evidence of the relationship between the results of FCB-DM screening and conventional venous blood pathology tests. Correlations were observed between a number of FCB-DM variables and corresponding venous blood pathology tests. Further findings indicated that the differential white cell counts as determined by FCB-DM were significantly correlated with that determined by conventional automated venous haematology for neutrophil, lymphocyte, monocyte and eosinophil populations. There were however some differences when comparing the absolute values of white cell counts between the two testing techniques.

In respect of the hypotheses tested:

- Null Hypothesis A was rejected as significant correlations between fasting average platelet area and venous pathology platelet counts were observed.
- Null Hypothesis B was rejected because significant correlations between venous blood pathology ESR and FCB-DM fasting fibrin number and fasting fibrin area were observed.
- Null Hypothesis C was partially rejected as significant correlations were observed between FCB-DM fasting chylomicron remnant counts and venous blood pathology triglycerides and insulin, and between FCB-DM fasting chylomicron remnant counts and $HOMA_B$ and $HOMA_{IR}$ calculations. A significant correlation was also observed between postprandial chylomicron remnant counts and HbA1c but no significant correlations were observed between FCB-DM chylomicron remnant counts and HDL and LDL cholesterol or glucose levels. Statistically significant differences were observed between the FCB-DM fasting and postprandial chylomicron counts.
- Null Hypothesis D was rejected as statistically significant correlations were observed between FCB-DM neutrophil, lymphocyte, monocyte and eosinophil populations and venous pathology counts.

Despite the limitations that came to light in the course of this study it remains that our data has demonstrated moderate but significant correlations between FCB-DM screening and selected conventional venous blood pathology tests, that are utilised in clinical practice for metabolic syndrome diagnosis. FCB-DM has the

potential to provide a cost effective, point of care screening tool that is less invasive than venous blood pathology. Further research should strengthen the clinical value of FCB-DM screening in monitoring metabolic syndrome, as well as other disease states.

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7 Appendices

7.1 Appendix A. Published Paper.

A Comparison of Differential Leucocyte Counts Measured by Conventional Automated Venous Haematology and Darkfield Microscopic Examination of Fresh Capillary Blood

Advances in Integrative Medicine, accepted for publication available online June 3rd 2015.

Katrina Reeve[1], Jacinta Arellano[1], Tini Gruner[1], Wayne Reilly[2], Belinda Smith[1], Shi Zhou[1]

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[2]Wellness Care Australia

Abstract
Analysis of fresh capillary blood using darkfield microscopy (FCB-DM) is a point of care screening tool, used by healthcare practitioners in Australia. However, the relationship between the outcomes of FCB-DM measures and the automated venous haematology measures has not been determined.
Objectives: The aim of this study was to compare differential white blood cell counts obtained using automated haematology and FCB-DM.
Methods: Data of 125 individuals were collected either retrospectively (n=74) or from participants specifically recruited for the project (n=51). Retrospective data were collected from active files at a naturopathic clinic. Newly recruited participants provided a fasting capillary blood sample for FCB-DM analysis within one hour of providing a venous blood sample at a commercial laboratory for automated haematologic analysis.
Results: The mean score of neutrophils was found to be higher, and lymphocytes and basophils to be lower, in FCB-DM analysis ($p < 0.001$). A significant and positive Pearson's correlation coefficient was found between automated haematology and FCB-DM in the cell counts for neutrophils (r =0.60, p <0 .001) and lymphocytes (r = 0.63, $p < 0.001$), and a significant and positive Spearman's correlation was found for monocytes (r_s = 0.32, $p <$ 0.001) and eosinophils (r_s =0 .596, $p < 0.001$). Linear regression analysis was also conducted to assess the relationship between the two techniques. The variance explained by the regression model was large for neutrophil (37%), lymphocyte (39%), and eosinophil (37%) scores.
Conclusion: Despite significant differences in the mean scores of cells counts, significant correlations between the data obtained by the two techniques for neutrophil, lymphocyte, monocyte and eosinophil cells were observed. Given the small amount of blood sample required, FCB-DM would have an advantage in clinical practice, though further research is required to determine the clinical implications of the FCB-DM cell counts.

Key words: leucocyte, capillary blood, microscopy, differential count, screening, haematology

7.2 Appendix B. Abstract, Oral Presentation, AHMRC 2014.

Is darkfield microscopic analysis of fresh capillary blood a viable alternative option to venous haematology for health screening? (19928)

Katrina Reeve [1] Jacinta Arellano [1] Wayne Reilly [2] Shi Zhou [1]

- *Southern Cross University, Lismore*
- *Wellness Care Australia, Brisbane*

Fresh capillary blood analysis using darkfield microscopy (FCB-DM) is a point of care screening tool used by complementary healthcare practitioners in Australia and New Zealand. In this technique, capillary blood samples taken from a fingerpick are left untreated and viewed immediately in their fresh state under a darkfield microscope.

Aim: The aim of this study was to investigate the relationship between various blood parameters using FCB-DM and automated venous blood haematology including the differential white blood cell count.

Methods: Data were collected retrospectively (n = 74) using existing patient files as well as blood samples taken from subjects specifically recruited for the project (n= 51). The cohort included both men and women between the ages of 24 and 70 with BMI ranges from 16.8 to 55. Ethics Approval was received May 7th 2013 (ECN-13-111).

Results: The differential white blood cell counts determined by the two techniques were compared for each participant. A significant positive correlation was found between the two techniques in neutrophil (r =0.60, p <0 .001), lymphocyte (r = 0.63, p < 0.001), monocyte (r_s = 0.32, p < 0.001) and eosinophil (r_s =0 .596, p < 0.001) cell populations.

Conclusion: These results show a statistically significant relationship between neutrophil, lymphocyte, monocyte and eosinophil cell counts performed by FCB-DM and conventional venous blood haematology. This supports and justifies the use of this technique in clinical practice, as a quick and cost effective way of monitoring leukocyte populations.

7.3 Appendix C. Poster, MEPSA 2013.

Identification of abnormal lymphocytes during routine screening using Hemaview™ – a case study

Katrina J Reeve[1] and Wayne G Reilly[2]
Southern Cross University[1] , Wellness Care Australia Pty Ltd[2]

Background:

Hemaview™ is a darkfield microscopy technique that uses capillary blood to examine blood parameters such as erythrocyte and leukocyte morphology.

Hairy cell leukaemia is a chronic lymphatic leukaemia that involves clonal expansion of mature appearing lymphocytes. Lymphocyte accumulation begins in the bone marrow and spreads to the lymph nodes and other lymphoid tissues[1]. Seventy-five per scent of diagnosed cases are in patients over 60 years of age. Symptoms tend to be nonspecific fatigue, anorexia, easy bruising and bleeding, lymphadenopathy, weight loss, dyspnoea on exertion and abdominal fullness from an enlarged spleen or palpable lymph nodes. Diagnosis is usually based on abnormal blood counts and blood films, and confirmed through bone marrow aspirations.

Methodology:

Hemaview™ is a technique that uses a drop of peripheral blood from a finger prick using a sterile lancet device. The drop of blood is collected onto a glass Menzel-Glaser frosted microscope slide and a Menzel – Glasser, 22x40mm number 1.5 coverslip is carefully placed onto the drop of blood within a few seconds of collection. The blood was then examined using a Zeiss Optiphot darkfield microscope, with a darkfield oil condenser, using Achroplan objectives x40, x100 (iris oil immersion-na1.25) and Immersol 518 N immersion oil. A differential count was carried out using Hemaview™ computing software and images of white blood cells collected.



Figure 1. Heamview™ technique

Results:

An 84 year old woman presented with fatigue, weight loss, failure to thrive and bowel frequency following a mild cardiovascular episode. A routine Hemaview ™ was performed and lymphocytosis, monocytosis and blast cells were observed. The lymphocytes were of abnormal morphology, larger than standard with hair like projections. White cell differential counts showed 11% neutrophils, 72% lymphocytes, 13% monocytes, 2% eosinophils and 2% basophils.

The patient was referred to her GP for further investigations. Automated pathology counts and blood film confirmed a diagnosis of Hairy cell Leukaemia that was previously undiagnosed. The differential counts were 22% neutrophils, 63% lymphocytes, 13% monocytes, 1% eosinophils and 1% basophils.

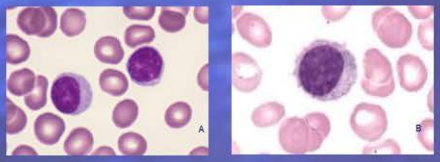


Figure 2 Normal lymphocytes (A) and Hairy cell lymphocyte (B) on blood film

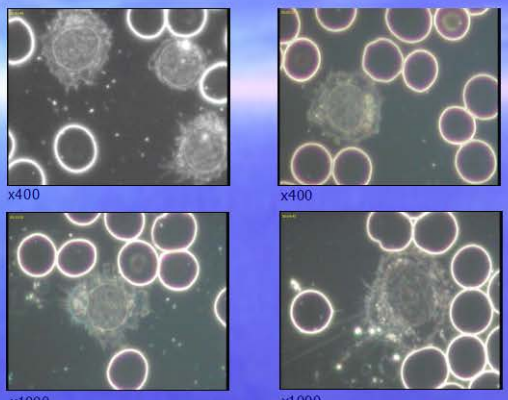


Figure 3: Images of abnormal lymphocytes as seen through Heamview™

	Neutrophils 1	Lymphocytes 2	Monocytes 3	Eosinophils 4	Basophils 5
Hemaview 1st Visit	42	42	11	2	0
Hemaview 2nd Visit	11	72	13	2	2
Hemaview 3rd Visit	45	46	6	1	2
Pathology at time of 2nd Visit	22	63	13	1	1

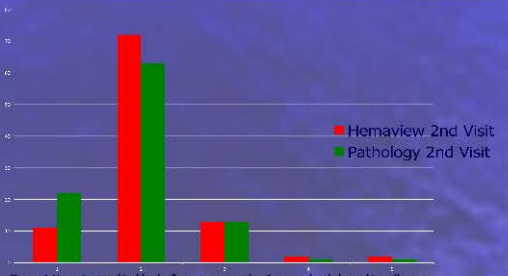


Figure 4 Heamview™ white blood cell counts compared to Automated pathology white cell counts

Conclusion:

The white blood cell differential counts between the automated analysis and Hemaview™ in this case are statistically similar. Further more morphological examination of white blood cells with Hemaview™ facilitated improved patient care. This case study is further justification of the clinical utility of Hemaview™.

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7.4 Appendix D. Abstract, Oral Presentation, MEPSA 2011.

Hemaview™: A Clinically Useful Tool for White Blood Cell Differential Counts

Hemaview ™ is a darkfield microscopy technique that uses capillary blood to examine blood parameters such as erythrocyte and leukocyte numbers and morphology. It has been widely used in Australia, USA and Europe for more than 20 years and can be performed rapidly at the point of care, allowing for fast and effective patient treatment.

Differential white blood cell counts can be performed using Hemaview ™ and theoretically, give similar results to blood films and automated pathology tests. It is recognised that the automated systems are faster and cost effective for counting of white blood cells, red blood cells and platelets, whereas visual microscopy is superior for differentiating cells based on nuances of cytological features, especially for immature cells.

This study investigated how white blood cell differential counts performed with Hemaview ™ correlate to automated white blood cell counts.

Data that was collected from a Naturopathic clinic in Brisbane during 2010 will be analysed. Cases selected require automated blood pathology to have been taken within 4 days of Hemaview ™ being performed.

Due to advances in technology, automated haematology analysers can provide precise and accurate white blood cell differential counts in most blood samples. However, when abnormal cells are present a manual differential count or examination of a stained blood film is still required. Hemaview ™ provides a fast and cost effective technique to analyse blood in a clinical setting.

7.5 Appendix E. Waist Circumference Thresholds.

Current recommended waist circumference thresholds for abdominal obesity by organisation (K. G. Alberti et al., 2009)

		Recommended Waist Circumference Threshold for Abdominal Obesity	
Population	Organization (Reference)	Men	Women
Europid	IDF (4)	≥94 cm	≥80 cm
Caucasian	WHO (7)	≥94 cm (increased risk)	≥80 cm (increased risk)
		≥102 cm (still higher risk)	≥88 cm (still higher risk)
United States	AHA/NHLBI (ATP III)* (5)	≥102 cm	≥88 cm
Canada	Health Canada (8,9)	≥102 cm	≥88 cm
European	European Cardiovascular Societies (10)	≥102 cm	≥88 cm
Asian (including Japanese)	IDF (4)	≥90 cm	≥80 cm
Asian	WHO (11)	≥90 cm	≥80 cm
Japanese	Japanese Obesity Society (12)	≥85 cm	≥90 cm
China	Cooperative Task Force (13)	≥85 cm	≥80 cm
Middle East, Mediterranean	IDF (4)	≥94 cm	≥80 cm
Sub-Saharan African	IDF (4)	≥94 cm	≥80 cm
Ethnic Central and South American	IDF (4)	≥90 cm	≥80 cm

*Recent AHA/NHLBI guidelines for metabolic syndrome recognize an increased risk for CVD and diabetes at waist-circumference thresholds of ≥94 cm in men and ≥80 cm in women and identify these as optional cut points for individuals or populations with increased insulin resistance.

7.6 Appendix F. Causes of Neutropenia and Neutrophilia.

Neutrophilia or increased numbers of neutrophils in the blood can be caused by:
• Bacterial infections • Inflammation and tissue necrosis, post myocardial infarction • Metabolic disorders such as gout, eclampsia, uraemia, acidosis • Neoplasms (carcinoma, lymphoma, melanoma) • Acute haemorrhage or haemolysis • Medications such as corticosteroid therapy, lithium, tetracycline • Myeloid proliferative diseases, chronic myeloid leukaemia, polycythaemia vera, myelofibrosis, essential thrombocythaemia • Treatment with myeloid growth factors • Asplenia • Pregnancy • Smoking
Neutropenia or decreased numbers of neutrophils in the blood can be caused by:

• Kostmann's syndrome
• Medications such as
  o Anti-inflammatory drugs – phenylbutazone
  o Antibacterial drugs – chloramphenicol, sulfasazine, imipenm
  o Anticonvulsants – phenytoin, carbamazepine
  o Antithyroids – carbimazole
  o Hypoglycaemics – tolbutamide
  o Phenothiazines – chlorpromazine, thioridazine
  o Psychotropics and anti-depressants – clozapine, mianserin, imipramine
  o Miscellaneous – gold, penicillamine, mepacrine, frusemide, deferiprone
• Autoimmune conditions, systemic lupus erythematosus
• Large granular lymphocytic leukaemia
• Viral infections
• Fulminant bacterial infections – typhoid, military tuberculosis
• Bone marrow failure
• Splenomegaly

(Hoffbrand, 2006, p. 117; Metagenics, 2010)

7.7 Appendix G. Recruitment Letter.

Dear Colleague,

I am conducting a research project as part of a Masters program through Southern Cross University. This project is looking into the use of live capillary blood analysis (Hemaview™) in the screening for metabolic syndrome, insulin resistance and inflammation.

We are seeking participants for this study from the following groups:

Patients with a high waist measurement - Women above 88 cm (> size 16); Men above 102 cm (> 42" pants) *OR*
Patients with a healthy weight measurement – Women below 80 cm (< size 10); Men below 94 cm (< 34" pants)

The project will involve about 3 hours of the participant's time in which fasting venous blood will be taken at a QML collecton centre, followed by two Hemaview™ analyses performed two hours apart (fasting and postprandial) at Cooraparoo Integrated Healthcare Centre in Brisbane or Southern Cross University in Lismore. We will also be performing blood glucose measurements and body composition analysis and asking the participant to fill out a detailed questionnaire.

A full report including the pathology and Hemaview™ results will be given to the participants to help inform their future healthcare. Venous testing will include: Full Blood Examination including Liver Function Tests, ESR/ CRP, Iron studies, Lipid studies and Triglycerides (incl. Apo A/B), Blood glucose (fasting), HbA1c.

Participation in this project will provide a cost effective opportunity for a detailed health check for elegible participants.

If you or any of your patients or friends would be interested in being involved, I would appreciate you passing on these details. I can be contacted on:
03 3394 3711 # 0410 459 447
katrina321@bigpond.com

Thank you in advance for your time and support in this valuable research project.

Yours Sincerely,

Katrina Reeve
Naturopath
BA, BHSc (Naturopathy), Dip Opera, LTCL, Cert IV Msg

7.8 Appendix H. Southern Cross University Recruitment Email.

Would you like to know more about your health?

Hello, My name is Katrina Reeve and I am conducting a research project as part of a Masters program through the School of Health and Human Sciences, under the supervision of Prof. Shi Zhou and Dr Jacinta Arellano.

I am investigating a fresh capillary blood analysis technique that is currently used in Naturopathic and Medical clinics in Australia.

Participation will require you to give a sample of blood for standard pathology blood tests at a QML collection centre, followed by a few drops of fingertip blood for live blood testing (taken over a few hours) on campus in Lismore or at a clinic in Brisbane. Participants will receive copies of the pathology and blood screening results to help inform their future healthcare. Participation in this project is free, and would provide a cost effective opportunity for a detailed health check for eligible participants.

We are seeking participants with waist measurements:

- For women above 88 cm (> size 16) *OR* below 80 cm (< size 10)
- For men above 102 cm (> 42" pants) *OR* below 94cm (< 34" pants)

So if you are interested in finding out more information about this project please contact:

Katrina Reeve

Email: kreeve.12@student.scu.edu.au

Phone: 0410 458 447

This research has been approved by the Southern Cross University Human Research Ethics Committee Approval # ECN-13-111.

7.9 Appendix I. Raw Data Table – Fibrin and Platelets.

Platelets and Fibrin		Venous blood pathology			FCB-DM Platelets		FCB-DM Fibrin		
Location	Participant ID number	Platelets	ESR	CRP	Fasting average platelet number	Fasting average platelet area	Fasting average fibrin cluster number	Fasting average fibrin cluster area	Fasting diffuse fibrin 0=none 1=mild 2=moderate 3=severe
Brisbane	1	251	5	1.9	4.1	330.5	0.0	0.0	0.0
Brisbane	2	174	36	7	4.1	414.4	0.2	316.3	0.9
Brisbane	5	262	5	2.5	2.3	215.1	0.3	366.7	0.4
Brisbane	7	201	5	1.9	2.9	139.9	0.1	37.5	1.1
Brisbane	8	181	5	0.1	9.3	260.4	0.1	23.1	0.1
Brisbane	10	257	22	14	7.7	331.8	0.0	0.0	0.3
Brisbane	11	196	5	0.1	2.3	290.7	0.0	0.0	0.0
Brisbane	13	203	16	0.9	5.2	295.9	0.1	182.9	1.1
Brisbane	16	330	8	12	3.1	594.9	0.1	214.9	0.5
Brisbane	17	181	5	0.3	9.0	203.6	0.0	0.0	0.0
Brisbane	18	251	8	5	6.6	353.3	0.0	0.0	0.0
Brisbane	19	189	19	3.3	4.5	301.1	0.1	55.3	0.6
Brisbane	20	218	5	4.9	4.8	228.7	0.0	0.0	0.3
Brisbane	22	192	8	0.8	4.9	254.5	0.7	6.8	0.0
Brisbane	27	249	24	1.8	1.2	85.9	0.0	0.0	0.0
Brisbane	29	304	11	12	4.5	797.9	0.1	85.2	1.2
Brisbane	30	366	16	9	2.3	301.5	0.0	0.0	0.1
Brisbane	31	223	8	3.6	8.8	185.6	0.0	0.0	0.0
Brisbane	33	240	8	5.9	4.3	338.4	0.0	0.0	0.1
Brisbane	35	186	8	4.9	1.4	140.9	0.1	24.4	0.0
Brisbane	36	251	5	4.9	6.5	341.2	0.0	0.0	0.0
Brisbane	40	204	8	0.4	6.8	158.1	0.0	0.0	0.0
Brisbane	41	235	5	n/a	2.7	241.7	0.0	0.0	0.7
Brisbane	42	281	14	4.9	6.2	408.4	0.4	1115.3	2.3
Brisbane	45	199	5	0.5	2.7	263.7	0.0	0.0	0.8
Brisbane	47	162	11	4.9	6.3	222.7	0.0	0.0	0.0
Brisbane	48	186	2	0.1	5.1	286.4	0.0	0.0	0.0
Brisbane	49	315	24	0.2	3.8	410.6	0.0	0.0	0.5
Brisbane	51	257	5	0.1	9.8	292.7	0.0	0.0	0.0
Brisbane	52	282	8	4.5	7.1	345.8	0.1	17.6	0.3
Brisbane	55	233	22	7	3.5	574.6	0.5	1303.3	1.2
Brisbane	57	201	11	1.8	3.5	385.6	0.0	0.0	0.1
Brisbane	59	212	22	4.9	8.9	232.5	0.0	0.0	0.0
Brisbane	63	193	5	4.9	4.3	149.3	0.0	0.0	0.0
Brisbane	65	227	5	4.9	6.1	312.1	0.3	942.6	1.3
Brisbane	69	274	22	4.9	5.9	272.7	0.1	338.2	0.1
Brisbane	70	n/a	8	n/a	8.5	226.9	0.0	0.0	0.0
Brisbane	72	243	19	n/a	3.4	347.9	0.1	95.1	0.7
Brisbane	73	279	5	0.1	5.8	447.4	0.0	0.0	0.0

Platelets and Fibrin		Venous blood pathology			FCB-DM Platelets		FCB-DM Fibrin		
Location	Participant ID number	Platelets	ESR	CRP	Fasting average platelet number	Fasting average platelet area	Fasting average fibrin cluster number	Fasting average fibrin cluster area	Fasting diffuse fibrin 0=none 1=mild 2=moderate 3=severe
Brisbane	74	358	14	9.5	5.7	287.7	0.2	300.0	1.9
Brisbane	78	267	5	4.9	4.3	153.3	0.0	0.0	0.4
Brisbane	80	198	2	0.4	10.8	202.0	0.1	94.9	0.6
Brisbane	81	266	16	0.6	5.0	240.1	0.0	0.0	0.0
Brisbane	84	259	5	4.2	9.5	311.5	0.5	1015.8	1.1
Brisbane	86	239	11	4.5	5.5	470.1	0.4	1821.9	0.9
Brisbane	90	242	14	4.9	8.2	318.0	0.3	254.3	0.5
Brisbane	92	308	14	4.9	11.3	206.1	0.0	0.0	0.0
Brisbane	94	231	14	4.9	7.7	134.7	0.0	0.0	0.0
Brisbane	99	229	24	1.7	5.3	305.9	17.0	0.0	0.1
Brisbane	79	266	5	4.9	9.2	231.5	0.0	0.0	0.0
Lismore	201	252	16	4.9	5.0	79.0	0.0	0.0	0.7
Lismore	202	210	5	0.3	7.0	122.0	0.1	11.0	1.1
Lismore	203	326	29	4	9.0	274.0	0.0	0.0	0.7
Lismore	204	213	5	4.9	3.0	77.0	0.0	0.0	0.0
Lismore	205	237	2	0.1	7.0	122.0	0.0	0.0	0.0
Lismore	206	272	11	4.9	6.0	26.0	0.0	0.0	0.6
Lismore	207	175	32	4.9	4.0	18.0	0.0	0.0	1.3
Lismore	208	266	5	1.4	8.0	29.0	0.0	0.0	0.0
Lismore	209	186	2	0.1	2.0	26.0	0.0	0.0	0.0
Lismore	210	282	11	0.1	4.0	34.0	0.2	10.0	0.7
Lismore	211	285	5	0.8	2.0	22.0	0.0	0.0	0.0
Lismore	212	345	16	5.2	6.0	27.0	0.0	0.0	0.0
Lismore	213	201	8	4.9	5.0	136.0	0.0	0.0	0.0
Lismore	214	267	19	22	3.0	39.0	0.1	47.0	1.0
Lismore	215	250	11	0.1	6.0	23.0	0.0	0.0	0.0
Lismore	216	214	14	0.1	4.0	36.0	0.0	0.0	0.0
Lismore	217	367	2	18	9.0	30.0	0.0	0.0	0.0
Lismore	218	278	5	5	12.0	35.0	0.0	0.0	0.0
Lismore	219	311	5	4.9	6.0	34.0	0.0	0.0	0.0

7.10 Appendix J. Raw Data Table – Chylomicron Remnants.

Chylomicron remnants		Venous blood pathology							FCB-DM	
Location	Participant ID number	BMI	HOMA-B	HOMA-IR	HbA1C	Fasting glucose	Fasting insulin	Fasting triglycerides	Fasting average chylomicron remnants	Postprandial average chylomicron remnants
Brisbane	1	23.4	5	1.9	5.5	5.6	5	1.3	7.4	109.0
Brisbane	2	43.9	36	7	6.1	6.6	26	2.6	15.4	111.3
Brisbane	5	28.9	5	2.5	5.8	5.8	7	0.9	1.1	88.9
Brisbane	7	32.2	5	1.9	5.7	6.3	20	2	15.2	79.7
Brisbane	8	23.5	5	0.1	5	3.9	4	0.6	8.7	34.8
Brisbane	10	49	22	14	5.4	5.3	16	1.1	48.0	49.9
Brisbane	11	22.1	5	0.1	5.2	4.5	8	0.4	15.0	15.5
Brisbane	13	20.1	16	0.9	5.6	4.8	7	0.7	1.3	142.3
Brisbane	16	35	8	12	5.4	5.5	18	1	8.1	76.4
Brisbane	17	38.3	5	0.3	5.6	5.9	34	1.5	50.9	92.5
Brisbane	18	29.5	8	5	5.4	5.8	8	1.4	63.8	90.5
Brisbane	19	37.1	19	3.3	5.4	5.4	10	0.5	34.9	36.3
Brisbane	20	21.5	5	4.9	5.1	4.8	6	0.6	20.5	115.1
Brisbane	22	20.2	8	0.8	5	4.9	6	0.4	4.5	24.0
Brisbane	27	22.3	24	1.8	5.9	5.4	4	0.5	1.9	103.5
Brisbane	29	39.1	11	12	4.9	5.2	18	1	33.5	65.0
Brisbane	30	40.1	16	9	5.7	5.3	19	5.9	50.4	53.2
Brisbane	31	47.7	8	3.6	5.8	5.4	21	0.6	29.1	52.5
Brisbane	33	29.7	8	5.9	4.7	5.2	16	0.4	11.1	54.5
Brisbane	35	22.7	8	4.9	5	4.9	3	0.4	0.5	23.3
Brisbane	36	21.6	5	4.9	5.2	5.3	3	0.4	33.6	111.8
Brisbane	40	26.6	8	0.4	5.2	5.4	4	0.4	19.9	26.6
Brisbane	41	34.9	5	3.94	6	6.5	31	2.3	35.8	60.7
Brisbane	42	55	14	4.9	6.2	5.5	10	1.6	9.3	134.7
Brisbane	45	31.1	5	0.5	5.9	6.3	31	1.4	18.1	77.0
Brisbane	47	16.8	11	4.9	5.9	4.3	3	0.5	12.8	124.4
Brisbane	48	20	2	0.1	5.3	4.2	3	0.4	5.1	77.1
Brisbane	49	21.6	24	0.2	5.6	5.6	6	0.8	6.9	110.3
Brisbane	51	30.2	5	0.1	5.3	6.3	9	2.4	30.3	70.2
Brisbane	52	31.3	8	4.5	5.5	5.4	10	0.9	13.2	44.1
Brisbane	55	44.9	22	7	5.4	5.2	18	1.1	9.2	53.9
Brisbane	57	39.3	11	1.8	6.3	6	28	2.5	12.8	68.3
Brisbane	59	34.7	22	4.9	5.6	6.5	18	1.1	25.4	160.2
Brisbane	63	21.8	5	4.9	5	5.9	6	0.3	5.0	68.3
Brisbane	65	31.8	5	4.9	5.5	5.7	17	1.4	50.5	52.2
Brisbane	69	19.7	22	4.9	5.5	4.9	2	0.5	3.3	90.0
Brisbane	70	24.2	8	n/a	5.6	4.9	n/a	n/a	7.3	109.0
Brisbane	72	34.5	19	1.4	5.5	5.3	11	1.4	0.7	31.3
Brisbane	73	21.4	5	0.1	5.1	5.3	5	0.9	8.4	125.3

Chylomicron remnants		Venous blood pathology							FCB-DM	
Location	Participant ID number	BMI	HOMA-B	HOMA-IR	HbA1C	Fasting glucose	Fasting insulin	Fasting triglycerides	Fasting average chylomicron remnants	Postprandial average chylomicron remnants
Brisbane	74	43.1	14	9.5	5.2	5.2	20	1.2	4.1	23.1
Brisbane	78	19.3	5	4.9	5.5	4.8	2	0.3	22.5	85.1
Brisbane	80	23.9	2	0.4	5.7	6.3	6	0.8	103.3	131.8
Brisbane	81	21.2	16	0.6	5.5	5.4	4	0.5	2.9	92.7
Brisbane	84	33.4	5	4.2	5.2	5	25	1.5	49.0	88.8
Brisbane	86	32.8	11	4.5	5.4	5.4	14	0.5	7.5	84.2
Brisbane	90	40.4	14	4.9	5.2	5.4	12	1	0.7	58.7
Brisbane	92	26.9	14	4.9	5.7	6	18	0.5	26.1	110.5
Brisbane	94	20.9	14	4.9	5.3	4.3	3	0.4	10.1	97.1
Brisbane	99	24.7	24	1.7	5	5.5	14	0.8	30.6	45.7
Brisbane	79	21.6	5	4.9	5	4.3	4	0.7	194.0	73.0
Lismore	201	22	16	4.9	5.6	4.4	6	0.7	6.0	32.0
Lismore	202	24.3	5	0.3	5.3	5.6	4	0.9	57.0	67.0
Lismore	203	20	29	4	5.4	5	7	0.5	44.0	95.0
Lismore	204	21.8	5	4.9	5.5	4.9	14	0.4	9.0	81.0
Lismore	205	23	2	0.1	5.4	4.9	7	0.8	7.0	74.0
Lismore	206	21.8	11	4.9	5.1	4.3	6	0.3	5.0	45.0
Lismore	207	33	32	4.9	5.8	5.4	16	1	7.0	167.0
Lismore	208	34.3	5	1.4	5.5	5	13	0.5	14.0	58.0
Lismore	209	20.3	2	0.1	5.7	5.1	8	0.4	7.0	68.0
Lismore	210	24	11	0.1	5.2	4.3	5	0.5	145.0	53.0
Lismore	211	28	5	0.8	5.7	5.3	15	0.9	32.0	82.0
Lismore	212	28.7	16	5.2	5.6	5.5	8	1.8	5.0	45.0
Lismore	213	21.3	8	4.9	5.4	4.1	6	0.3	16.0	94.0
Lismore	214	36.1	19	22	5.5	5	13	1.7	12.0	71.0
Lismore	215	17.9	11	0.1	5.1	4.6	5	0.6	22.0	65.0
Lismore	216	29.6	14	0.1	5.5	4.7	11	0.8	7.0	46.0
Lismore	217	42.8	2	18	5.2	4.5	14	0.6	34.0	54.0
Lismore	218	45.4	5	5	5.3	5.1	11	0.8	20.0	31.0
Lismore	219	27.6	5	4.9	5.7	4.4	12	1.3	14.0	66.0

7.11 Appendix K. Ethics approvals.

Southern Cross University

Division of Research

HUMAN RESEARCH ETHICS COMMITTEE (HREC)
HUMAN RESEARCH ETHICS SUB-COMMITTEE (HRESC)

NOTIFICATION

To: Dr Jacinta Arellano/ Katrina Reeve/Dr Tini Gruner
School of Health and Human Sciences
k.reeve.12@student.scu.edu.au;tini.gruner@scu.edu.au;jacinta.arellano@scu.edu.au

From: Secretary, Human Research Ethics Committee
Division of Research, R Block

Date: 7 May 2013

Project name: *An investigation into the validity of darkfield microscopic analysis of fresh capillary blood as a screening tool for inflammation and metabolic disease.*

Approval Number ECN-13-111

The Southern Cross University Human Research Ethics Committee has established, in accordance with the National Statement on Ethical Conduct in Human Research – Section 5/Processes of Research Governance and Ethical Review, a procedure for expedited review and ratification by a delegated authority of the HREC.

Thank you for your expedited ethics application dated the 29 April 2013. This has been considered low risk by the Acting Chair, Dr David Lloyd and has been approved.

All ethics approvals are subject to standard conditions of approval.

These should be noted by researchers as there is compliance and monitoring advice included in these conditions.

Ms Sue Kelly
HREC Administration
T: (02) 6626 9139
E: ethics.lismore@scu.edu.au

Dr David Lloyd
Chair, *pro tem*, HREC
E. david.lloyd@scu.edu.au

Southern Cross University

HUMAN RESEARCH ETHICS COMMITTEE (HREC)
HUMAN RESEARCH ETHICS SUB-COMMITTEE (HRESC)

NOTIFICATION

To: Dr Tini Gruner / Katrina Reeve
School of Health and Human Sciences
k.reeve.12@scu.edu.au,tini.gruner@scu.edu.au

From: Secretary, Human Research Ethics Committee
Division of Research, R. Block

Date: 10 January 2012

Project: **A comparison between automated pathology and live blood analysis using darkfield microscopy of differential white blood cell counts.**

Approval Number: ECN-12-002

The Southern Cross University Human Research Ethics Committee has established, in accordance with the National Statement on Ethical Conduct in Human Research – Section 5/Processes of Research Governance and Ethical Review, a procedure for expedited review and ratification by a delegated authority of the HREC.

This has been considered and approved by the Chair of the HREC, Professor Bill Boyd.

This approval is subject to the usual standard conditions of approval. Please see over.

Helen Wolton
HREC Administration
Ph: (02) 6626 9139
E. ethics.lismore@scu.edu.au

Professor Bill Boyd
Chair, HREC
Ph: 02 6620 3569
E. william.boyd@scu.edu.au

7.12 Appendix L. Correlation results

Brisbane			Lismore		
Automated haematology	**FCB-DM**	**Correlation**	**Automated haematology**	**FCB-DM**	**Correlation**
Platelets			**Platelets**		
Platelet count	Fasting platelet area	r_s (50) = .36, p < .05*	Platelet count	Fasting platelet area	r (19) = .11, p < .65
Platelet count	Fasting platelet number	r (50) = .03, p < .831	Platelet count	Fasting platelet number	r (19) = .03, p < .91
CRP	Fasting platelet area	r (50) = .44, p < .05*	ESR	Fasting platelet area	r (19) = .26, p < .29
ESR	Fasting platelet number	r (50) = .13, p < .37	ESR	Fasting platelet number	r (19) = .01, p < .96
Fibrin			**Fibrin**		
ESR	Fasting fibrin area	r (51) = .49, p < .05*	ESR	Fasting fibrin area	r (19) = .47, p < .05*
ESR	Fasting fibrin number	r (51) = .69, p < .05*	ESR	Fasting fibrin number	r (19) = .42, p > .05*
BMI	Fating diffuse fibrin	r (51) = .56, p < .05*	CRP	Fasting fibrin area	r (19) = .11, p > .05
BMI	Postprandial diffuse fibrin	r (51) = .44, p < .05*	CRP	Fasting fibrin number	r (19) = .09, p > .05
Chylomicron remnants					
Triglycerides	Fasting chylomicron remnant	r_s (50) = .30, p < .05*			
HOMA B	Fasting chylomicron remnant	r (47) = .31, p < .05*			
HOMA IR	Fasting chylomicron remnant	r (46) = .31, p < .05*			
Insulin	Fasting chylomicron remnant	r (50) = .33, p < .05*			
HbA1c	Postprandial chylomicron remnant	r_s (51) = .35, p < .05*			

* Significant at .05