CIRCULATING CELLS ASSOCIATED WITH CARDIOVASCULAR OUTCOMES IN PERIPHERAL ARTERIAL OCCLUSIVE DISEASE

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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Medicine).

Declaration

I, *David Terence Martin*, declare that the material presented in this thesis is my own work. All information derived from the work of others, published or unpublished, has been acknowledged in the text and references given. Assistance and guidance of others in preparing this thesis has been duly noted in the statement of the contribution of others.

This work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution.

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Dr David Terence Martin Date

Dedication

To my family

My Grandparents, who I wish could have seen this completed:

Terrence Joseph Martin (22/8/1923 – 7/7/2012)

Thelma Barbara McKay (18/1/1929 – 13/11/2003)

John ‘Jack’ McKay (23/11/1927 – 2/12/2006)

My parents, who I credit with my past, present and future success:

Dennis Joseph Martin

Barbara Martin

My brother and sister, who support me with everything:

Simon Martin

Miranda Sainsbury

My wife, for her love and understanding:

Murissa Martin

And my son who provided the motivation for the completion of this thesis:

Edward James Martin

Statement of Access

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Chapter	Contributions (indicated by initials)
Overall Project	Original concept: JG / DM Review of collected data: DM / CR Compilation of thesis: DM
Chapter 1: Introduction to circulating cell counts in peripheral arterial occlusive disease	Writing chapter: DM Proofreading of chapter: DM / MC / CR
Chapter 2: Aims, Objectives and Hypothesis	Concept: DM /JG Writing chapter: DM Proofreading of chapter: DM / MC / CR
Chapter 3: Association of total white cell count with mortality and major adverse events in patients with peripheral arterial occlusive disease: a systematic review	Concept: DM / JG Methodology: DM / JG Data collection: DM Independent screening of papers: DM / DW Data analysis: DM Figures/Tables: DM Writing publication: DM Proofreading of publication: DM / DW / MC / CR / PT/ JG
Chapter 4: Study design and methodology	Concept: JG / DM Data collection: DM Figures/Tables: DM Writing chapter: DM Proofreading of chapter: DM / MC / CR / JG
Chapter 5: Results section 1 Cohort characteristics	Concept: DM Methodology: DM / JG Data collection: DM Kaplan Meier data analysis: DM Figures/Tables: DM Writing chapter: DM Proofreading of chapter: DM / MC / CR
Chapter 6: Results section 2 Cohort incidence of cardiovascular events in patients with peripheral arterial occlusive disease	Concept: DM Methodology: DM / JG Data collection: DM Kaplan Meier and Log Rank data analysis: DM Figures/Tables: DM Writing chapter: DM Proofreading of chapter: DM / MC / CR

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Chapter 7: Results section 3 Disease severity associated with major adverse events in patients with peripheral arterial occlusive disease	Concept: DM Methodology: DM / JG Data collection: DM Kaplan Meier and Log Rank data analysis: DM Figures/Tables: DM Writing chapter: DM Proofreading of chapter: DM / MC / CR
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I agree that the prior statements about my respective contributions to authorship are true:

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Ethics Declaration

The research study contained within this thesis received research ethics approval from James Cook University Human Research Ethics Committee (approval H2196, Appendix A), Mater Health Services North Queensland Human Research Ethics Committee (Appendix B) and Townsville Health Services District Human Research Ethics Committee (approval 61/05, Appendix C). All participants included in this study signed a participant informed consent form (Appendix D or Appendix E) and received a participant information sheet (Appendix F or Appendix G).

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Publications and presentations

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Martin, D., Crowe, M., Rush, C., Tosenovsky, P., Golledge, J. Predicting outcomes in patients with PVD/ PAOD. North Queensland Regional Vascular Meeting 01/06/2013 Townsville

Martin, D. Predicting outcomes in patients with PVD/PAOD. North Queensland Regional Vascular Meeting 1/6/2013 Townsville.

Poster Presentations:

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Martin, D., Crowe, M., Rush, C., Tosenovsky, P., Golledge, J. Association of total white cell count with mortality and major adverse events in patients with peripheral arterial disease: A systematic review. Poster presentation at The Townsville Health Research Week 2013.

Other work submitted during candidature

Publications:

Martin, D., Smith, R.K., Velu, R., (2014). Delayed pseudoaneurysm following below knee amputation. J Vasc Surg. 62 (2) 489 + cover.

Makhija, N., **Martin, D**., Velu, R., (2014). Asymptomatic mirror right aortic arch. ANZ Journal of Surgery. 85 (9), 687-688.

Robinson, B. M., **Martin, D**., Velu, R.., & Yadav, S. (2012). Partial descending thoracic aortic replacement for chronic Type B dissection. Heart, Lung and Circulation, 21(11), 740-742.

Wallace, D., Woolley, T., **Martin, D**., Rasalam, R., Bellei, M., (2017) Medication calculation and administration workshop and hurdle assessment increases student awareness towards the importance of safe practices to decrease medication error in the future. Education for Health. 29(3), 171-178.

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Presentations:

Martin, D., Wallace. D., Raslam, R. Preventing medication errors through simulating medication calculation and administration. Presentation SIMHEALTH national conference 2014.

Martin, D., Bellei, M., Raslam, R., Harvey, N., Wallace, D., Contribution of simulation-based medical education to work-readiness of JCU graduates. Presentation at SIMHEALTH national conference 2014.

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Martin, D. Casemix and complications 2011. North Queensland Regional Vascular Meeting 19/5/2012 Townsville

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Martin, D., Smith R.K., Velu, R. Symptomatic partially thrombosed abdominal aortic aneurysm – endovascular management. Poster presentation at North Queensland Festival of life Sciences 2013.

Martin, D., McKenna, C., Velu, R. Covered stenting of a large popliteal pseudoaneurysm. Poster presentation at North Queensland Festival of Life Sciences 2013.

Martin, D., Candy, N., Velu, R. Nutcracker syndrome causing haematuria. Poster presentation at North Queensland Festival of Life Sciences 2013.

Martin, D., Velu, R. Splenic artery aneurysm in a young woman. Poster presentation at North Queensland Festival of Life Sciences 2013.

Martin, D., Tosenovsky P. Central Venous Occlusion from Artero-Venous fistula. Poster presentation at North Queensland Festival of Life Sciences 2013.

Martin, D., Smith, R.K., Velu, R.. Delayed pseudoaneurysm following below knee amputation. Poster presentation at North Queensland Festival of Life Sciences 2013.

Martin, D., Smith R.K., Marney, L., Velu, R. North Queensland thoracic aortic injury – endovascular treatment. The chimney graft approach in a high risk polytrauma setting. Poster presentation at The Townsville Health Research Week 2013

Marney, L., **Martin, D**., Sitharthan, S. & Velu, R. Traumatic thoracic aortic injury tackled with the chimney graft technique: A novel approach in a high risk poly-trauma setting. Poster presentation RACS 82nd Annual Scientific Congress 2013.

Smith, R.K., **Martin, D**., Velu, R. Delayed pseudoaneurysm following below knee amputation. Poster presentation at North Queensland Festival of Life Sciences 2013.

Wallace, D., **Martin, D**., Wooley, T., Rasalam, R. Decreasing medication errors though Doctor education. Poster presentation at North Queensland Festival of Life Sciences 2013.

Smith, R.K., **Martin, D**., Velu, R. Delayed pseudoaneurysm following below knee amputation. Poster presentation at The Townsville Health Research Week 2013.

Smith, R.K., **Martin, D**. Dengue fever in a rural hospital: issues concerning transmission. Poster presentation at The Townsville Health Research Week 2013

Smith, R.K., Wyke, D., **Martin, D**., Niles, N. Perforator anatomy of the anterolateral thigh free flap: a systematic review. AHNS 9th International Conference on Head and Neck Cancer 16-20 July 2016 Seattle, Washington

Smith, R., Quigley, F. **Martin, D**., Tosenovsky, P., Velu, R., Bradshaw, B., Buettner, P., Golledge, J. Severity of chronic venous disease and total white blood cell count. Poster Presentation ANZSVS 2014.

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Rodrigues, B., Gilbotra, R., Vengavati, V., Porter, D., **Martin, D**., Golledge, J., Malabu, U. Pattern of major lower limb amputations at The Townsville Hospital: a retrospective review. Poster presentation at The Townsville Health Research Week 2013.

Abstract

Background: Peripheral arterial occlusive disease is a manifestation of the inflammatory disease atherosclerosis, characterised by reduced blood flow to the limbs by narrowing and blocking of arteries. This disease affects more than 200 million adults worldwide and is a powerful indicator of widespread arterial disease. It is associated with increased risk of death, heart attack and stroke. Models to predict which patients with peripheral arterial occlusive disease will suffer these outcomes are lacking. Traditional null hypothesis testing methods to develop outcome models for this population are limited by the complexity of variable interaction and some variables exhibiting small although clinically important effects. The Information-Theoretical approach and particularly multi-model analysis and inference has been applied in other scientific fields but has not been applied to the population of patients with peripheral arterial occlusive disease.

Objective: The specific aims of this study were (1) to determine the association of the circulating cells of inflammation with clinical disease severity and traditional risk factors for the composite end-point of major adverse event consisting of death, heart attack or stroke and death alone using traditional null hypothesis testing statistics (Kaplan-Meir survival analysis and Cox proportional hazards analysis) compared to the novel approach of multi-model analysis; (2) to generate a predictive model for these cardiovascular outcomes in patients with peripheral arterial occlusive disease.

Design: Longitudinal cohort design with 632 patients considered and 398 patients included.

Methods: Patients were prospectively recruited from 2002 to 2014 from The Townsville Hospital and Townsville Mater Hospital and followed up until death, discharge from clinic or the conclusion of data collection on 1/12/2014. The blood sample for this study was obtained at recruitment if the patient was well, or otherwise at greater than one month post interventional procedure, major adverse event or resolution of infection. Kaplan-Meir analysis was performed for each endpoint and Cox proportional hazards analysis was used to test the association of each of the circulating cell types (total white cell count and its subsets neutrophils, lymphocytes, monocytes, the calculated neutrophil lymphocyte ratio and haemoglobin) with the endpoint of major adverse event and death *a priori* and with adjustment for risk factors. Further analysis was undertaken using a novel approach of multi-model analysis to generate an average best fit model for major adverse event and death.

Results: Disease severity was significantly associated with major adverse event using Kaplan-Meier and log rank analysis. Cox proportional hazards analysis for each cell type with major adverse event demonstrated high total white cell count, high neutrophil count and high monocyte count to be significantly associated in all analyses with both mid and high lymphocyte counts significantly associated. For the outcome of death high total white cell count, high neutrophil count, and high neutrophil/lymphocyte ratio were significantly associated in all analyses with high lymphocyte count protective. The importance of adjusting for traditional risk factors, disease severity and medication use in Cox proportional hazards analysis was demonstrated. Both the average best fit models from multi-model averaging for major adverse event and death feature clinical disease severity and circulating cell counts as stronger predictors than the risk factors traditionally associated with the establishment of the disease. High monocyte category was the cell type with the strongest positive influence in the

model for major adverse event, with high lymphocyte count having the strongest negative (i.e. protective) influence. The model for the outcome of death showed the disease severity categories of tissue loss and rest pain as having the strongest influence with high neutrophil count the strongest influencing cell type, and again high lymphocyte count having the strongest protective influence. Methods to enable the clinician to apply the model at the bedside or clinic are discussed using information from clinical history, disease severity and full blood count alone.

Conclusion: The Information-Theoretic approach of multi-model averaging is more appropriate and meaningful than traditional null hypothesis testing approaches for patients with peripheral arterial occlusive disease. Disease severity and circulating cell counts better discriminate patients at high risk of major adverse events and death than traditional risk factors. Circulating cell counts may be modelled with disease severity and traditional risk factors to guide treatment selection, aid patient compliance with lifestyle change and medical therapy, drive future pathophysiological research and generate potential treatments for patients with peripheral arterial occlusive disease.

Key words:

Peripheral arterial occlusive disease, peripheral arterial disease, leucocytes, total white cell count, death, mortality, major adverse event, multi-model averaging.

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Key Terms and Abbreviations

AAA	Aortic Abdominal Aneurysm
ABI	Ankle brachial index
ACE	Angiotensin converting enzyme inhibitor (antihypertensive)
ADEP	Atherosclerotic Disease Evolution by Picotamide study
AIC	Akaike information criterion
AICc	Sample size corrected Akaike information criterion
AngII	Angiotensin II receptor antagonist (antihypertensive)
APACHE	Acute Physiological and Chronic Health Evaluation score
ARIC	Atherosclerosis Risk in Communities
AUSLAB	Queensland Health Clinical and Scientific Information System
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CAPRIE	Clopidogrel versus aspirin in patients at risk of adverse ischaemic events
CAS	Carotid artery stent
CCB	Calcium channel blocker
CEA	Carotid endarterectomy
CHF	Congestive heart failure
CI	Confidence Interval
CLI	Critical Limb Ischaemia
CABG	Coronary Artery Bypass Grafting
Cox2	Cox 2 receptor selective non-steroidal anti-inflammatory drug

CRF^2	Comprehensive risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
CRP	C reactive protein
CVD	Cerebrovascular disease
DF	Degrees of Freedom
DM	Diabetes Mellitus
ECG	Electrocardiogram
Endo	Endovascular
F	Fontaine classification system
GAS	Glasgow Aneurysm Score
getABI	German Epidemiological Trial of Ankle Brachial Index
Hb	Haemoglobin
HbA1C	(measure of glycated haemoglobin – specifically the beta-N-1-deoxy fructosyl component of haemoglobin)
HDL	High Density Lipoprotein
HMG-CoA	Hydroxy-methyl-glutaryl coenzyme A
HR	Hazard ratio
HTN	Hypertension
IHD	Ischaemic heart disease
LC	Lymphocyte count
LDL	Low Density Lipoprotein
LEB	Lower extremity bypass
LES	Lower extremity stent

MAE	Major adverse event (death, heart attack or stroke)
MI	Myocardial infarction (heart attack)
MI/IHD	Myocardial infarction (heart attack) or ischaemic heart disease
MOOSE	Meta-analysis of observational studies in epidemiology
NHANES	National Health and Nutrition Examination Survey 1999-2002
n/a	Not applicable
NC	Neutrophil count
NLR	Neutrophil lymphocyte ratio
NR	Not reported
NS	Not significant
NSAIDs	Non-steroidal anti-inflammatory drugs
OR	Odds ratio
PACK	Prevention of Atherosclerotic Complication with Ketanserin study
PAD	Peripheral arterial disease
PAOD	Peripheral arterial occlusive disease
POSSUM	Physiological and Operative Severity Score for the enUmeration of Mortality and morbidity
PCI	Percutaneous coronary intervention
Prev Endovascular	Previous endovascular intervention
Prev Open	Previous open revascularisation
PRISMA	Preferred reporting items for systematic reviews and meta-analysis
PTA	Percutaneous transluminal angioplasty
QML	Queensland Medical Laboratories
RR	Relative Risk

REACH	Reduction of Atherothrombosis for Continued Health
SE	Standard error
SOLVD	Studies of Left Ventricular Dysfunction
SQL	Structured query language
strata	Statistical stratification for other significant categorical variables
SVE	Significant vascular event
T/AAA	Open thoracic or abdominal aortic aneurysm repair
T/EVAR	Thoracic or abdominal endovascular aneurysm repair
TIA	Transient ischaemic attack
TRF[1]	Traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
TWCC	Total white cell count
VBOHM	Vascular Biochemical and Haematological Outcome Model
WBC	White Blood Cell
WCC	White cell count
WHO	World Health Organisation
w	Akaike weight

1. Introduction to circulating cell counts in peripheral arterial occlusive disease.

Inflammation of the arteries causes peripheral arterial occlusive disease which has limb and life threatening implications. Models to better predict patients at high risk of major adverse cardiovascular events are required to add to known information from traditional risk factors and disease severity to enable patient tailored treatment and increase patient compliance with lifestyle changes and medical management.

1.1. Background

1.1.1. Definition and prevalence of peripheral arterial occlusive disease

Peripheral arterial occlusive disease is one manifestation of the inflammatory disease atherosclerosis, characterised by reduced blood flow to the limbs caused by arterial stenosis and occlusions. Peripheral arterial occlusive disease affects between 10 and 29% of people over the age of 55 years with prevalence increasing with age,[1-9] and has been reported as high as 33% in populations with diabetes mellitus.[10] Peripheral arterial occlusive disease affects an estimated 27 million adults in Europe and North America alone,[11,12] and an estimated 202 million adults worldwide.[13] Atherosclerosis is associated with the main causes of mortality on a worldwide scale with the absolute number of deaths from these causes continuing to increase.[14]

Symptomatic peripheral arterial occlusive disease ranges in severity from intermittent claudication (reproducible pain in the lower limbs on exercise that resolves with rest) to critical limb ischaemia (rest pain and/or ulceration or tissue loss – gangrene).[12] Patients with peripheral

arterial occlusive disease often report a reduced quality of life from the reduction in walking distance and speed which limits mobility and independence.[15-18] A large proportion of the patients with peripheral arterial occlusive disease are asymptomatic[1] (for every one symptomatic there are three to four asymptomatic patients with peripheral arterial occlusive disease)[5,8] although these patients are exposed to similar elevated cardiovascular risk.[19]

Peripheral arterial occlusive disease is a manifestation of the systemic inflammatory disease of atherosclerosis, a multi-factorial disease that affects all of the arterial systems of the body, principally involving the medium and large elastic and muscular arteries and can cause ischaemia to the end organs of the body including heart, brain, intra-abdominal organs and the tissues of the lower limbs. The presence of peripheral arterial occlusive disease is an indicator of diffuse and significant arterial disease and has been associated with an increased risk of cardiovascular and cerebrovascular events including death, myocardial infarction, stroke, and peripheral vascular intervention including major amputation.[11,12,20,21] Traditional risk factors for ischaemic vascular diseases do not explain all epidemiological features of these diseases.[22]

1.1.2. Natural history of peripheral arterial occlusive disease

Peripheral arterial occlusive disease is pathologically progressive however the presentation and symptoms of peripheral arterial occlusive disease patients do not necessarily follow a linear trajectory. Traditionally the severity of peripheral arterial occlusive disease has been classified with the Rutherford and Fontaine classification systems (Appendix I). Of the patients with symptomatic peripheral arterial occlusive disease who present with intermittent claudication (reproducible leg pain on exercise that improves with rest) only one quarter will clinically deteriorate with worsening claudication or the development of critical limb ischaemia.[23,24]

Despite this relatively small proportion of patients with intermittent claudication progressing clinically, this same patient group has 5 year mortality of 10-15%, with the majority (75%) dying from cardiovascular causes.[25] Within the same time a further 20% of this population will sustain a non-fatal heart attack or stroke.[25] Of the 1-3% of patients who present with critical limb ischaemia, 20-25% are dead within 12 months[26,27] while a further 30% undergo major amputation.[28] The 10 year mortality in the critical limb ischaemia population is 75%.[26] A flow diagram representing the clinical progression of peripheral arterial occlusive disease and cardiovascular outcomes adapted from Norgren et al.[28] is presented in Figure 1.1.

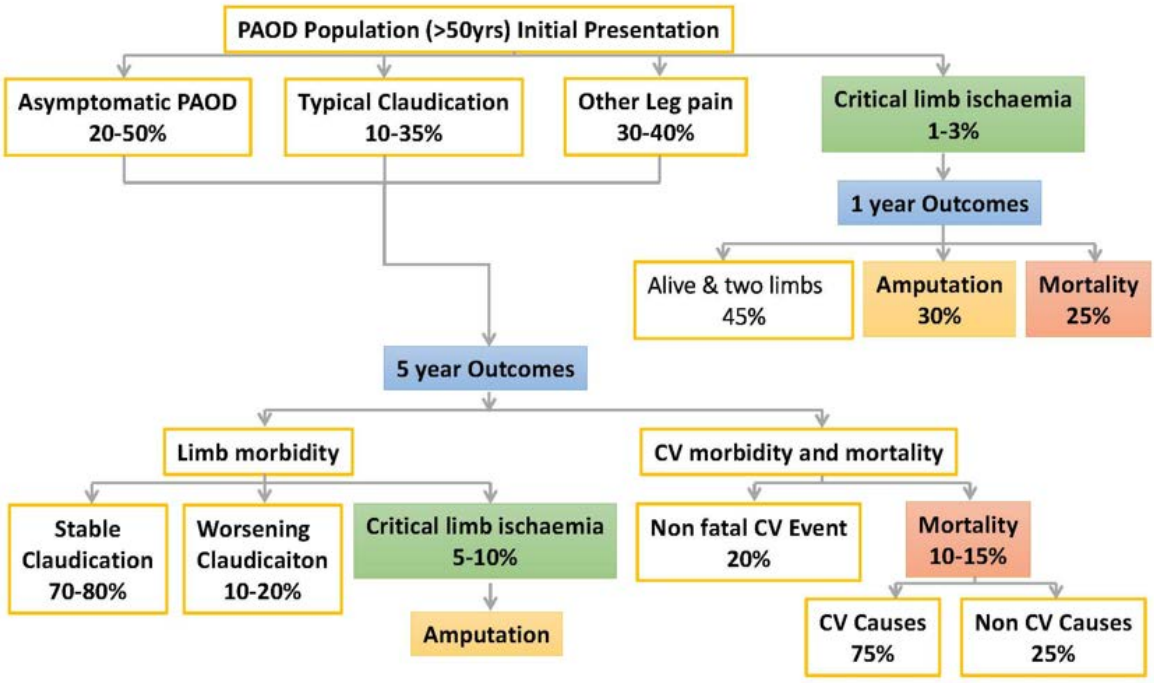

Figure 1.1: Flow Diagram of clinical progression of peripheral arterial occlusive disease

Adapted from Norgren et al. [28]
PAOD = Peripheral Arterial Occlusive disease
CV = Cardiovascular

Coronary artery disease, cerebrovascular disease, and peripheral arterial occlusive disease are all manifestations of atherosclerosis and therefore commonly occur together. Peripheral arterial

occlusive disease is a powerful indicator of diffuse and significant atherothrombotic disease.[21,29,30] In the REACH (Reduction of Atherothrombosis for Continued Health) registry containing 67 888 patients more than one half of the patients with peripheral arterial occlusive disease had demonstrated atherosclerotic disease in another arterial territory (coronary or cerebral)[31] with the peripheral arterial occlusive disease subpopulation demonstrating the highest one year atherothrombotic event (cardiovascular death, heart attack, stroke, or hospitalisation for a cardiovascular event) rate of 21.1% which was greater than patients with coronary artery disease (15.2%) or cerebrovascular disease (14.5%).[32] In the first year of this registry 10% of peripheral arterial occlusive disease patients required lower limb intervention and 1.6% required major amputation.[32] The higher risk of adverse cardiovascular events in patients with peripheral arterial occlusive disease is clinical importance and the ability of clinicians to be able to accurately assess this risk and predict major adverse events should affect both conservative and interventional management of this patient population. The primary endpoint of this study will be major adverse event: a composite outcome of death, heart attack or stroke.

1.1.3. Peripheral arterial occlusive disease mortality implications

Patients with peripheral arterial occlusive disease have an increased risk of subsequent heart attack and stroke and are 6 times more likely to die from cardiovascular disease within 10 years than patients without peripheral arterial occlusive disease.[29] Patients with symptomatic peripheral arterial occlusive disease have a 15yr accrued survival rate of approximately 22% compared to a survival rate of 78% in patients without peripheral arterial occlusive disease.[28]

Peripheral arterial occlusive disease patients frequently suffer from concomitant coronary artery disease and cerebrovascular atherosclerosis and therefore are at a particularly high risk for cardiovascular complications.[3,28,33] In the REACH study the risk of major adverse events for patients with peripheral arterial occlusive disease was twice as high in the patients that had involvement of more than one vascular bed.[31] While commonality exists in risk factors for the development of atherosclerotic disease in all arterial beds, smoking, hypertension and diabetes appear most important risk factors for the development of peripheral arterial occlusive disease[13], compared to hypertension and lipid profile being a more important factor in the development of coronary artery disease. Despite these differences atherosclerosis may affect multiple arterial beds in patients with peripheral arterial occlusive disease, in one population study, patients with peripheral arterial occlusive disease had ischaemic heart disease as a comorbidity in 58% and cerebrovascular disease in 34%.[33] The method of detection of coronary artery disease in the peripheral arterial occlusive disease population greatly affects the reported prevalence with variation from 19% to 90% of patients with peripheral arterial occlusive disease diagnosed as also having coronary artery disease with >90% being diagnosed as having coronary artery disease if angiography or autopsy is used.[34-36]

The presence of peripheral arterial occlusive disease has been associated with an increased risk of cardiovascular and cerebrovascular events including death, myocardial infarction, stroke, and peripheral vascular intervention including major amputation.[12,20,21,23,29,30,33,37,38] In the Framingham study almost 70% of the deaths in the patients with intermittent claudication were due to a cardiovascular cause.[39] Patients with peripheral arterial occlusive disease have been reported to have a higher incidence of major adverse event than patients with isolated coronary artery disease or cerebrovascular disease.[32] The relative risk of fatal and non-fatal

cardiovascular events in patients with peripheral arterial occlusive disease is 3.1 of death and 5.9 of death from cardiovascular disease times that of control patients after adjustment for age and cardiovascular risk factors.[29,30] Similar results have been demonstrated in other populations.[40,41] The clinical severity of peripheral arterial occlusive disease has been associated with higher mortality with severe symptomatic patients experiencing 15 fold increase in mortality rate in the San Diego population study equating to 75% 10 year mortality.[29]

Patients with peripheral arterial occlusive disease are medically managed to reduce this risk of cardiovascular morbidity and mortality at significantly lower rates than patients with ischaemic heart disease despite comparable mortality.[42,43] The increased mortality risk of patients with peripheral arterial occlusive disease once established is less clearly related to traditional risk factors, Brevetti et al. (2010)[44] proposed that the subsequent cardiovascular risk is related to the severity and extent of the underlying atherosclerotic disease and possibly other factors including inflammation.

1.1.4. Inflammation

Inflammation has long been suspected of playing a central role in the development and progression of systemic atherosclerosis.[45-48] Atherosclerosis is increasingly understood to be an active, inflammatory process in which the circulating inflammatory cells have integral roles.[46,47,49,50] No longer a lipid storage disease, inflammatory cells are involved not only in the initiation and evolution of atheroma with dysfunctional endothelium but in the acute thrombotic complications of atheroma arising from exacerbated inflammation and plaque rupture.[46,51,52]

The longitudinal relationship between inflammation and peripheral arterial occlusive disease has been established.[53-57] Inflammation is associated with the presence,[53] progression[54] and severity of peripheral arterial occlusive disease.[58,59] The risk of major adverse cardiovascular event in patients with peripheral arterial occlusive disease is less clearly related to traditional risk factors than in patients with ischaemic heart disease or cerebrovascular disease[29,38,60,61] and may be more related to the systemic inflammation.[21,62-68] Increasing evidence indicates that inflammatory parameters are associated with the risk of future ischaemic events.[69] While systemic inflammation plays a role in peripheral atherosclerosis over and above the traditional risk factors, markers of inflammation have not been inconsistent as a predictive tool.[44,59,63,70,71] Combining inflammatory markers with peripheral arterial occlusive disease severity and traditional risk factors improves risk stratification[59,65]

Few studies have investigated the prognostic implications of circulating cells and adverse events in peripheral arterial occlusive disease.[24,72-74] The need to assess the relationship between systemic inflammation and cardiovascular risk was identified by Brevetti et al.[44] who recommended following a sufficiently large population longitudinally with measures of systemic inflammation, traditional risk factors and measures of peripheral arterial occlusive disease severity. Low grade systemic inflammatory response leads to an increased number of circulating neutrophils and decreased lymphocyte count.[75-77] Assessment of circulating cell counts as a marker of systemic inflammation is inexpensive and already routinely obtained pre-operatively, although it's correlation with cardiovascular outcomes in patients who present with peripheral artery disease has not been firmly established.

1.2. Risk factors for outcome in peripheral arterial occlusive disease

The presented study will assess the association and predictive value of circulating total and differential cell counts with cardiovascular outcomes in peripheral arterial occlusive disease patients and will compare these to the traditional risk factors for peripheral arterial occlusive disease in the development of models to predict cardiovascular outcomes. The precedent for the inclusion of each risk factor will be discussed separately.

1.2.1. Age

Age is a standard factor included in predictive models and is important in the peripheral arterial occlusive disease population as an increase in both incidence and prevalence of peripheral arterial occlusive disease has been shown with increasing age. [1,28] Mortality is a known function of increasing age.[78]

1.2.2. Sex

Cardiovascular mortality in both asymptomatic and symptomatic peripheral arterial occlusive disease is significantly higher in men.[79] Prevalence of both symptomatic and asymptomatic peripheral arterial occlusive disease is higher in men than in women with the ratio increasing in more severe stages of the disease,[1,2,80] Although, a higher incidence of women with critical limb ischaemia has been repoted.[9] Cardiovascular mortality in both asymptomatic and symptomatic peripheral arterial occlusive disease is significantly higher in men.[79]

1.2.3. Smoking status

Smoking is a strong risk factor for the development of peripheral arterial occlusive disease.[81,82] Smoking promotes the progression of peripheral arterial occlusive disease, increases rates of heart attack and stroke, increases risk of both graft occlusion and major amputation and worsens survival.[8,83-85] The strong association between smoking and elevated total white cell count has been documented in many studies,[22,86-91] and even exposure to second hand smoke in the "ATTICA study" (from the province of Attica in Greece which included Athens) was associated with elevated total white cell count.[92] Smoking increases the risk and reduces the success of peripheral vascular intervention.[80] The mechanisms by which smoking increases risk in peripheral arterial occlusive disease patients may be through increasing circulating white blood cell aggregation and reducing the deformability of cells, predisposing to microvasculature plugging and subsequent damage from the release of proteolytic enzymes and free radicals damaging the vessel endothelium, promoting further thrombosis and vessel occlusion.[93]

1.2.4. Diabetes mellitus

The Framingham study[94] showed a 3.5 fold risk among men and 8.6 fold risk among women with diabetes of developing peripheral arterial occlusive disease. The diabetic population has poorer outcomes from peripheral revascularisation[95] and requires major amputation at a rate five to fifteen times higher than non-diabetics.[28,96,97] Large population studies have shown than diabetic patients with peripheral arterial occlusive disease have 3-4 times the mortality risk of their healthy controls.[95,98] Diabetes is associated with elevated total white cell count.[87,89,90,99]

1.2.5. Hypertension

Hypertension is associated with all forms of cardiovascular disease,[28] and hypertension is an independent predictor of mortality.[100-103] Hypertension increases the risk of peripheral arterial occlusive disease[28] and peripheral arterial occlusive disease increases the risk of mortality,[24,104] but whether hypertension is an independent risk factor for mortality within the population with established peripheral arterial occlusive disease remains unclear.

1.2.6. Ischaemic heart disease

In the PARTNERS (Peripheral Arterial Disease Awareness, Risk and Treatment: New Resources for Survival) study over 50% of outpatients with peripheral arterial occlusive disease had a history of coronary artery disease.[5] In the REACH registry the one year cardiovascular event (cardiovascular death, heart attack, stroke or cardiovascular related hospitalisation) rate of patients with both ischaemic heart disease and peripheral arterial occlusive disease was 23.1% compared to 17.4% in those patients with peripheral arterial occlusive disease alone. Ischaemic heart disease is a predictor of short and long term mortality in vascular patients undergoing revascularisation or aneurysm repair.[105]

1.2.7. Stroke

A combination of stroke or transient ischaemic attack (TIA) was one of the identified risk factors that predicted mortality at 5 and 10 years in a review of mortality in 2642 consecutive peripheral arterial occlusive disease patients.[106] Touze et al.[107] quantified the risk of heart attack and non-stroke vascular mortality to ~2% per year following stroke or TIA.

1.2.8. Disease severity

The clinical stage of peripheral arterial occlusive disease as described by Rutherford and Fontaine (Appendix I) is important to include in outcome modelling because rate of major adverse event in patients with critical limb ischaemia approaches 20% at one year and 40-70% at 5 years[28,108] and their all cause and cardiovascular mortality has been reported as three times that of patients who present with intermittent claudication.[64] In the San Diego population study[29] severe symptomatic patients experienced 15 fold increase in mortality rate equating to 75% 10 year mortality, similar to the 80% 10 year mortality of Dormandy et al.[34] with rest pain.

When ankle-brachial index is used as an objective measure of the severity of peripheral arterial occlusive disease an ankle-brachial index <0.9 has been shown to predict all-cause mortality with a relative risk ratio of 3.8[109] 5 year all-cause mortality (Hazard Ratio 1.4)[110] and stroke with hazard ratio 2.5 for fatal stroke[111] Peripheral arterial occlusive disease patients with an ankle-brachial index <0.5 demonstrate twice the rate of mortality of claudicants with an ankle-brachial index >0.5.[24] Mehler[104] showed a 10% increase in relative risk of major adverse event for each 0.1 decrease in ankle-brachial index. Similar results were generated by Resnick[20] who also showed ankle-brachial index >1.4 to have a similar increase in mortality rate. The German Epidemiological Trial on Ankle Brachial Index (getABI) which included 6880 unselected patients from the primary care setting demonstrated that patients with an ABI <0.5 had a 31.4% 5 year all-cause mortality while in the patients with an ABI >1.1 mortality in the same time frame was 8.6%.[110,111]

While ankle-brachial index has been proposed to be included in models of outcome for patients with peripheral arterial occlusive disease,[19,81,112,113] the relationship with mortality and in-particular the cut points that should be used remain unclear. McDermott[114] did not demonstrate a significant difference in the survival of patients with an ankle-brachial index 0.31-0.49 when compared to ankle-brachial index 0.50-0.92 after adjustment for traditional risk factors but did demonstrate a lower cumulative survival in patients with an ankle-brachial index <0.30. Other studies have failed to demonstrate an association between ankle-brachial index and non-fatal cardiovascular events.[19,115,116] In this study the clinical severity of disease (Appendix I) was used and not ankle-brachial index.

1.2.9. Aspirin and HMG-Co-A reductase inhibitor (Statin) use

The Antithrombotic Trialists Collaboration have demonstrated that patients with cardiovascular disease experience a 25% odds reduction in subsequent major adverse events by the use of aspirin.[117] Aspirin use has been associated with significantly lower total white cell count in four separate non-randomised studies of cardiovascular risk[86,88,87,118] involving a total of >160 000 patients although these trials were not specifically designed to investigate this relationship and confounding variables may have influenced this apparent association.[99]

The JUPITER trial (Justification for the Use of statins in Prevention: an Intervention Trial Evaluating Rosuvastatin use) in a non-peripheral arterial occlusive disease population demonstrated a 44% reduction in all-cause and cardiovascular morality.[119] The Heart Protection Study reported that 40 mg Simvastatin daily in patients with peripheral arterial occlusive disease (even if not diagnosed with ischaemic heart disease) experienced 24% proportional reduction in major adverse events.[120,121] It is not surprising then that Statin use is

more common in survivors of the high risk peripheral arterial occlusive disease population with critical limb ischaemia at 12 months.[122] Short term statin use has been shown to reduce major adverse events within six months of vascular surgery.[123,124] Shillinger et al.[125] demonstrating that peripheral arterial occlusive disease patients receiving Statin therapy for a minimum of 4 weeks prior to endovascular peripheral intervention had significantly improved survival and event free survival rates, with the patients with the higher markers of inflammation benefiting the most from statin therapy.[125]

Statin use also favourably influences leg function,[126] and have been associated with infra-inguinal graft patency and limb salvage after infra-inguinal bypass surgery.[127] It remains unclear in the peripheral arterial occlusive disease population whether the reduced risk of major adverse events from statin use are due to the lipid lowering or anti-inflammatory effects.[125, 128]

1.2.10. Traditional Risk Factors for peripheral arterial occlusive disease in this study

In this study the risk factors of age, sex, smoking status, diabetes mellitus, hypertension, ischaemic heart disease, aspirin and statin use were recorded for all included participants. The contribution of these risk factors to mortality and major adverse event as potential confounding factors for the analysis of circulating cell counts is considered and addressed. These risk factors were also included in model formation for the outcomes of major adverse event and death.

1.3. Predictive models for outcome in patients with peripheral arterial occlusive disease

Current cardiovascular risk prediction in patients with peripheral arterial occlusive disease is limited to using equations attained through large observational studies such as Atherosclerosis

Risk in Communities (ARIC),[90,129] Framingham,[80,94] Honolulu[130] and Strong Heart[131] studies. These models have been generated using the traditional approach of null-hypothesis testing and stepwise regression analysis which has multiple limitations.[132] Further risk stratification has been proposed to include co-morbid disease, clinical stage of peripheral arterial occlusive disease, and haemodynamic severity of disease supplemented with biologic markers of inflammation.[133,134] The Information-Theoretical statistical paradigm[132] and particularly the multi-model averaging approach has not been previously applied to generate outcome models for patients with peripheral arterial occlusive disease.

In the population of patients with peripheral arterial occlusive disease who are at an established high risk of major adverse cardiovascular events a central goal of treatment is to reduce morbidity and mortality.[3] A risk stratification tool for patients with peripheral arterial occlusive disease would not only provide prognostic cardiovascular risk information but provide individual data that may assist clinicians to convey the importance of lifestyle modification and adherence to medical therapies.[133] This is clinically important as risk factor modification and adherence to medical therapies can reduce burden of disease.[135,136] Targeted smoking cessation has been shown to reduce mortality risk in peripheral arterial occlusive disease.[83,85]

Individualising outcome prediction rather than at population level will allow the identification of the patients within this population at highest risk and may allow for targeted interventions. Interventions including exercise training, claudication medications and selective revascularisation can improve or remove lower extremity ischaemic symptoms and may avoid lower limb amputation. Outcome modelling may assist clinicians in making decisions between open revascularisation, endovascular revascularisation, conservative management or primary

amputation.[137] Outcome predictive models have been developed for patients with coronary heart disease but are not currently available for patients with peripheral arterial occlusive disease.[133]

Outcome models have been developed for patients undergoing open abdominal aortic aneurysm (AAA) repair. The Glasgow Aneurysm Score (GAS)[138] was developed using logistic regression to identify significant factors determining post-operative outcome for open AAA repair. The GAS was prospectively validated among patients undergoing elective AAA repair[139] and has subsequently been retrospectively validated using large national database samples.[140-142] The GAS has been previously criticised that its low positive predictive value does not reliably identify high-risk patients and is inaccurate in predicting morbidity.[143,144] The Physiological and Operative Severity Score for the enUmeration of Mortality and morbidity (POSSUM) score[145] contains both physiological and operative components but can only be used to predict mortality pre-operatively if the physiological component is used alone.[146,147] Variations on the POSSUM model, the P-POSSUM[148,149] and V-POSSUM, have shown variable results when undergoing validation as a pre-operative predictive score.[143] Vascular Biochemical and Haematological Outcome Model (VBOHM) also used logistic regression using a minimal bloodwork panel dataset and although initially designed for audit[150] has been subsequently applied to elective and emergency AAA repair with good predictive value although there were problems with the calibration of the initial model.[143,151] VBOHM modelling has also been applied to describe outcomes following major amputation in critical limb ischaemia patients.[152] Other outcome models for AAA repair such as the Leiden score[153] which adjusts for centre specific mortality and the Lee Customised Probability Index for vascular surgical patients[154] which included the protective effect of statin use were both

problematic when applied to other populations (including endovascular repair of abdominal aortic aneurysms). The Acute Physiological and Chronic Health Evaluation scoring system (APACHE-AAA) has also been applied to this population with similar effect.[155]

To the authors knowledge no predictive outcome models have been applied to the peripheral arterial occlusive disease population with respect to outcomes of major adverse event and total mortality. The proposed model for investigation in the current study will combine traditional risk factors, disease severity, aspirin and statin use and circulating cell counts. Circulating cell counts are routinely obtained on full blood count analysis with an established WHO standard for measurement and excellent inter-assay precision. The aim is then to produce an accurate and reliable model using objective standardised measures which the clinician can easily and inexpensively apply at the bedside or in the outpatient clinic.

1.4. Circulating cell counts

The presented study will assess the association and predictive value of circulating total and differential cell counts with cardiovascular outcomes in peripheral arterial occlusive disease patients. The precedent for the inclusion of each cell type will be discussed separately.

1.4.1. Total white cell count

Atherosclerosis has been demonstrated to be a systemic inflammatory process and total white cell count provides an objective measure of inflammatory status.[50,156] The role that white blood cells play in this inflammatory processes as mediators of host defence and inflammation can be either acute or chronic and may be either reparative or maladaptive.[49] White blood cells may

contribute to the progression of vascular disease and the risk of subsequent major adverse event through physically plugging microvasculature, chemically mediating the inflammatory response through free radical production, or promoting thrombosis, all of which cause resultant endothelial cell dysfunction.[157,158] The white blood cell may be an underutilized predictive index in patients, suggesting which patients may be at an increased risk of cardiovascular complications related to a heightened systemic inflammatory response.

In population studies of apparently healthy participants total white cell count has been associated with the development of cardiovascular disease,[22,159] cardiovascular death,[160-166] and all cause mortality[160,161,165,167-169] even after adjusting for traditional cardiovascular risk factors.[147]

Meta-analysis by Danesh et al.[170] examined prospective cohort studies and demonstrated a combined risk ratio of 1.4 (1.3-1.5) of elevated total white cell count for the development of coronary heart disease in the largest seven studies investigating this relationship involving 5337 patients.

The relationship between total white cell count and heart attack in large population epidemiologic studies has been well published.[161,162,170-175] and persists even after adjustment for smoking and other traditional risk factors.[22,163,176]

Primary stroke[177-179] and death after stroke[180,181] have both been shown to have association with elevated total white cell count. Patients with high total white cell count have also been reported to have larger central nervous system lesions at presentation and more severe presenting

symptoms.[182] Smoking has long been associated with an elevated total white cell count [183-188]but the predictive value of total white cell count is not fully explained by this relationship.[171,189]

Rheologically active leucocytes play a pathophysiological role in atherogenesis through endothelial injury and adhesion with plugging of the microvascular circulation and induction of tissue ischaemia, propagated by increase in leucocyte number, further leucocyte attachment to the endothelium, and reduction in perfusion pressure causing ongoing positive feedback.[190,191] Whether the observed leucocytosis is causative or reactive to the underlying atherosclerotic process remains debated.[99,190,192]

Despite the advantages of being inexpensive, part of the routine laboratory assessment, being reliable with established World Health Organisation (WHO) standards[193] and excellent inter-assay precision, the impact of leukocyte count on cardiovascular risk is largely under investigated. This under-investigation is probably because of a tendency of both investigators and industry to focus on new assays and molecular mechanisms. [74,191] Total white cell count exhibits most of the characteristics desirable in a cardiovascular disease risk predictor described by Pearson et al.[194] in that it is standardised with the coefficient of variance of measurement <3% with established population norms; association with cardiovascular clinical end points has been established in observational studies and clinical trials; improves overall prediction of risk beyond that of traditional risk factors alone; is generalizable to various population groups; and is of acceptable cost. Total white cell count does not however meet the criteria of independence from other traditional risk factors and the interdependence on smoking needs careful consideration when assessing the role of total white cell count as a risk predictor

for major adverse events.[191] Coller[99] reported that inappropriate multivariate adjustments in previous investigations may have resulted in underestimating the true association of increased total white cell count with the increased risk of cardiovascular disease.

1.4.1.1. Total white cell count in ischaemic heart disease

The bulk of the published evidence on the association of total white cell count with death, heart attack and stroke in patients with atherosclerotic disease is from patients with known ischaemic heart disease. In patients with ischaemic heart disease total white cell count has been associated with higher mortality following heart attack[86,87,89,118,189,195-201] recurrent heart attack[189,202,203] and angiographic severity of coronary artery disease.[204] In the Evaluation of c7E3 for Prevention of Ischemic Complications (EPIC) study, the total WBC count correlated best with three year mortality.[205] The effect of total white cell count on mortality following acute heart attack may not be linear with evidence of a J-shaped relationship between mortality and total white cell count reported in large registry sudies[89,201,206] although this phenomena does not appear consistent in clinical trial data possibly due to the exclusion of patients with other medical conditions resulting in very low total white cell count.[205] In following up patients with unstable angina the incidence of major adverse event of patients with high total white cell count has been reported as much as eight times that of their counterparts with low total white cell count.[207] The excess risk of elevated total white cell count after heart attack may be at least in part attenuated by in-hospital revascularisation.[200,208] However, total white cell count has also been associated with mortality post coronary artery bypass grafting (CABG)[209-211] and percutaneous coronary intervention (PCI)[205,212-216] and stroke after CABG.[217] total white cell count has also been associated with new onset congestive cardiac failure.[87,89,218]

In the REACH registry a similar number of patients were reported as having both ischaemic heart disease and peripheral arterial occlusive disease to those who were identified as having peripheral arterial occlusive disease alone.[219] The true proportion of patients with peripheral arterial occlusive disease that also have coronary artery disease may be even higher, with >90% of patients with peripheral arterial occlusive disease having coronary artery disease if angiogram or autopsy is used for diagnosis.[34-36] The high prevalence coronary artery disease in the population of patients with peripheral arterial occlusive disease make the findings of total white cell count association with major adverse events in the coronary artery disease population very pertinent to the population of patients with peripheral arterial occlusive disease.

1.4.1.2. Total white cell count in peripheral arterial occlusive disease

Total white cell count has been associated with the presence of peripheral arterial occlusive disease in population studies. In the NHANES (National Health and Nutrition Examination Survey 1999-2002) total white cell count was associated with the presence of peripheral arterial occlusive disease as diagnosed by ankle-brachial index <0.9, with odds ratio of 1.67 for the top quartile (>7.3 $x10^9$ cells/L) compared to the bottom quartile (≤ 4.9 $x10^9$/L).[53] Total white cell count has also been shown to be related to the presence of carotid and femoral atherosclerosis.[220] Total white cell count along with elevated fasting plasma glucose level has been associated with asymptomatic peripheral arterial occlusive disease in a population of 125 patients with type 2 diabetes.[221]

Total white cell count has been proposed as the ideal marker of cardiovascular risk in peripheral arterial occlusive disease patients.[74] Total white cell count has been associated with an increased risk of cardiovascular event in patients with intermittent claudication,[24] patients

undergoing treatment for symptomatic peripheral arterial occlusive disease[74] and all-cause mortality in peripheral arterial occlusive disease patients.[24,222] Few studies have assessed the association of total white cell count with cardiovascular outcome in patients with peripheral arterial occlusive disease and these are addressed in detail in Chapter 3.[223]

Total white cell count has also been associated with adverse operative outcomes in patients with peripheral arterial occlusive disease, with higher total white cell counts in failed versus successful amputations in diabetic paitents,[224] incisional complications after infra-inguinal bypass surgery[225] and higher total white cell count has been used to predict femoral-popliteal bypass failure.[226]

1.4.2. Neutrophil Count

Neutrophils can adhere to the endothelium and facilitate plaque disruption by releasing superoxide radicals, proteolytic enzymes and arachidonic acid metabolites.[190,227,228] In addition neutrophils can aggregate with platelets and contribute to the microvascular and macrovascular plugging promoting infarction.[72,228,229] Neutrophils have also been implicated in adverse end-organ effects following ischaemic insult[230,231] possibly by the activation and reduced deformability of neutrophils induced by ischaemia[232] including the short term ischaemia of intermittent claudication.[233]

Neutrophil count was the major cell subtype that contributed to risk of heart attack in the Caerphilly study,[176] and elevated neutrophil count was significantly associated with the risk of recurrent ischaemic events in the Clopidogrel versus aspirin in patients at risk of adverse ischaemic events trial (CAPRIE) study[69] and Studies of Left Ventricular Dysfunction

(SOLVD) trials.[208] A large study by Horne et al.[229] which investigated 3277 patients with or at high risk for ischaemic heart disease showed that the high neutrophil quartile was a better predictor of death or heart attack than total white cell count. Neutrophil count has been associated with mortality following heart attack[205,234,235] and cardiovascular mortality.[236] Neutrophil count has also been shown to be significantly associated with the incidence of stroke in a Japanese cohort.[178] Constitutional neutropenia of Yemenite Jews appears to convey protection against atherosclerotic disease in an observational study.[237]

In patients with peripheral arterial occlusive disease, neutrophil count >5.8 $x10^9$cells/L increased risk for adverse events and added prognostic information to traditional atherothrombotic risk factors.[72] Neutrophil count >4.6 x 10^9cells/L associated with an increased risk of heart attack and stroke independent of confounding factors and cardiovascular treatment in 259 consecutive peripheral arterial occlusive disease patients.[74] Pre-procedural neutrophil count has been demonstrated to predict outcome in patients with peripheral arterial occlusive disease undergoing endovascular intervention.[238]

1.4.3. Lymphocyte Count

Lymphopenia has been described as being indicative of a generalized state of immunodepression with survival adversely influenced by the depressed immune function.[49] Described mechanisms of lymphopenia include margination and redistribution of lymphocytes within the lymphatic system and accelerated apoptosis.[99] Lymphopenia after major surgery has been associated with cortisol (a glucocorticoid hormone) production as a response to neuroendocrine stress and predisposes to post-operative complications.[239,240]

The protective effect of relative lymphocytosis has been observed in large studies[69,205,208] but the underlying mechanisms require further investigation. A large study by Horne et al.[229] which investigated 3277 patients with or at high risk for ischaemic heart disease, showed that the low lymphocyte quartile was a better predictor of death or heart attack than total white cell count. In the Clopidogrel versus aspirin in patients at risk of adverse ischaemic events (CAPRIE) study elevated lymphocyte count was inversely related with the risk of recurrent ischaemic events[69] with similar results in large studies of patients with ischaemic heart disease following coronary percutaneous intervention,[205] and Studies of Left Ventricular Dysfunction.[208]

In patients with peripheral arterial occlusive disease lymphocyte counts were reported as significantly higher in patients with critical limb ischaemia who had successful limb salvage after bone marrow implantation.[241]

1.4.4. Calculated: Neutrophil / Lymphocyte Ratio (NLR)

Neutrophil/lymphocyte ratio has been described as a reflection of the balance of the neutrophilia of inflammation and the relative lymphopenia of cortisol-induced stress response.[49] The study by Horne et al.[229] showed that the best predictor of death or heart attack was the calculated neutrophil/lymphocyte ratio. Neutrophil/lymphocyte ratio has a strong relationship within patients with coronary artery disease to adverse outcomes[229,242-246] following heart attack[227,243,247,248] in patients undergoing percutaneous intervention[212,249] and coronary bypass surgery.[250,251] Neutrophil/lymphocyte ratio has also been found to be a useful predictor of death, adverse outcomes and recurrence in Oncology patients.[75,252-259]

Pre-operative neutrophil/lymphocyte ratio identified patients at increased risk of death following major vascular surgery,[49] in patients with chronic critical limb ischaemia,[260] and predicted late mortality following thoracic endovascular aneurysm repair.[261] Elevated NLR has been proposed as a tool to identify patients with peripheral arterial occlusive disease at high risk of developing critical limb ischaemia.[262] Elevated NLR >3.0 has also been shown to be associated with a higher cardiovascular mortality in a cohort of 503 patients with symptomatic peripheral arterial occlusive disease.[263]

1.4.5. Monocyte Count

Ross described monocyte derived macrophages as mediating all the stages of atherosclerotic plaque formation and rupture.[47] Monocyte count in patients with ischaemic heart disease is associated with adverse cardiovascular events although to a lesser extent than neutrophil count.[99] Monocyte count associated with the presence of peripheral arterial occlusive disease in population studies.[264] Patients with peripheral arterial occlusive disease have monocytes that are more active than control groups when expression of CD40 and CD11b was compared.[265] Elevated monocyte count in patients with peripheral arterial occlusive disease has been associated with vein graft stenosis following lower limb bypass surgery but not mortality.[266] One study of 24 patients with critical limb ischaemia reported that elevated monocyte counts preoperatively diminish after successful revascularisation with bypass surgery.[267] Clear association of baseline monocyte counts in patients with peripheral arterial occlusive disease with cardiovascular endpoints remains to be established.

1.4.6. Eosinophil Count

Eosinophil count was associated with heart attack in the Caerphilly study[176] and eosinophilia predicted cardiovascular mortality in a population study with 30 year follow up in the

Netherlands.[268] In patients with coronary artery disease a more complex relationship between eosinophil count and mortality following PCI has been described, with an elevated eosinophil count associated with an improved outcome in the first 6 months after PCI but after this time being associated with increased mortality.[269] Clear associations of eosinophil counts with adverse cardiovascular events are yet to be established in patients with peripheral arterial occlusive disease therefore eosinophil counts were recorded but not analysed in the current study.

1.4.7. Basophil Count

No significant association between basophils and cardiovascular mortality has been reported despite inclusion in differential white cell count analysis in large studies. Therefore, basophil count was recorded but not analysed in the current study.

1.4.8. Haemoglobin

Low haemoglobin concentration may be an independent risk factor of cardiovascular risk in the general population[270] with the Framingham study demonstrating a U-shaped relationship between haematocrit and mortality.[271,272] Inflammation has been shown to cause immune driven depression of haematopoiesis in a manner roughly proportional to the duration and severity of disease.[273,274]

In coronary artery disease patients a low haemoglobin concentration has been shown to be a predictor of mortality after heart attack,[275] cardiac surgery,[276-280] PCI,[215,272,281,282] and in patients with heart failure.[270,283,284] The relationship does not appear to be linear with as many as 48% of the mortalities occurring in the lowest quintile of haemoglobin concentration.[281] Transfusion with red blood cells has been shown to reduce the mortality associated with low

haemoglobin concentration after heart attack.[272] Low haemoglobin concentration has also been associated with non-cardiac surgery mortality[285,286] and postoperative outcomes in elderly patients undergoing non-cardiac surgery.[287]

In patients with peripheral arterial occlusive disease, low haemoglobin concentration has been associated with <30 day mortality,[288] two year[49] and five year mortality following major vascular surgery independent of chronic renal disease and heart failure.[288] Low pre-operative haemoglobin has been shown to be a predictor of amputation site healing,[224] incisional complications,[225] and early loss of bypass patency.[289] Low haemoglobin has been associated with mortality in patients with asymptomatic carotid artery stenosis.[290] The relationship between haemoglobin and the composite endpoint of major adverse event remains unclear in patients with peripheral arterial occlusive disease.

1.5. Significance of this study

The clinical risk factors for the development of peripheral arterial occlusive disease have been well established,[1,25,28,31,37,73,80,82,94,129,134,219,291-295] and there is evidence that the severity of peripheral arterial occlusive disease is associated with the outcomes of death and major adverse event.[8,21,24,29,34,40,114,185,296,297] There is also evidence that circulating markers of inflammation in patients with peripheral arterial occlusive disease are associated with the outcomes of major adverse event and death.[24,69,72,74,160,161,165,167-169,223,263] However, which circulating markers of inflammation best contribute to models of outcome for patients with peripheral arterial occlusive disease remains unclear.

Models of outcome for patients with peripheral arterial occlusive disease have been proposed to include clinical risk factors, severity of disease and circulating markers of inflammation.[133,134] Models for outcome that combine these variables are not currently available for patients with peripheral arterial occlusive disease[133] despite their development for other manifestations of atherosclerosis such as coronary artery disease[298,299] and subsequent heart failure.[300]

Null hypothesis testing paradigm of statistical analysis has traditionally been used to infer associations for model creation and a selection of these methods were utilised in this study including Kaplan-Meir survival analysis with log-rank testing and Cox proportional hazards analysis. Null hypothesis testing techniques were used to establish the important variables associated with the outcomes of death and major adverse event and compare this study population to previously described populations. The limitations of the traditional null hypothesis testing approach are discussed, in particular the inadequacies of using techniques such as stepwise regression for model development. Stepwise regression and similar techniques are inherently compromised in populations with multiple interacting variables, especially if there are small but clinically important effects[301] such as in the population with peripheral arterial occlusive disease. Stepwise regression often results in the over or under estimation of variable strength,[302] missing true predictors[303] or the selection of less important predictors[303] depending on which variables are included in the model.[304,305] Null hypothesis testing techniques are not only unable to quantify the process that leads to final model selection but are unable to objectively compare competing models.[306]

The relatively modern statistical paradigm of Information-Theoretical analysis[132] is described with exploration of why this approach is more appropriate in the development of outcome models for patients with peripheral arterial occlusive disease. In this thesis the novel approach of Information-Theoretical based multi-model inference is applied to data from patients with peripheral arterial occlusive disease. Outcome models were subsequently developed for this population for the outcomes of major adverse event and death. Results of the multi-model analysis are presented including the development of an averaged best fit model for each outcome. To the authors knowledge this study is the first to comparatively assess traditional risk factors, clinical disease severity and circulating cell counts for the outcomes of death and major adverse event through the application of multi-model averaging from the Information-Theoretical statistical paradigm[132] which is widely used to in other scientific disciplines such as ecology and the behavioural sciences. This approach has enabled the generation of accurate and reliable models for the outcomes of major adverse event and death using clinical risk factors, clinical stage of disease and the circulating cell counts as markers of inflammation. The clinician can easily and inexpensively apply the developed models at the bedside or in the outpatient clinic with information routinely collected for these patients. The results of this model may be used to improve patient compliance with lifestyle change and medical therapy, risk stratify patients to aid in clinical decision making and guide future pathophysiological and therapeutic research.

2. Aim, Objectives and Hypothesis

The overall aim of this study was to develop an improved model to predict outcome in patients with peripheral arterial occlusive disease. The hypothesis that total and differential white cell count, haemoglobin and neutrophil lymphocyte ratio are associated with the incidence of major adverse event (composite outcome of death, heart attack or stroke) and death was tested by a cohort design study of prospectively recruited patients with symptomatic peripheral arterial occlusive disease. A schematic overview of the thesis is presented in Figure 2.1 and will be referred to throughout the thesis.

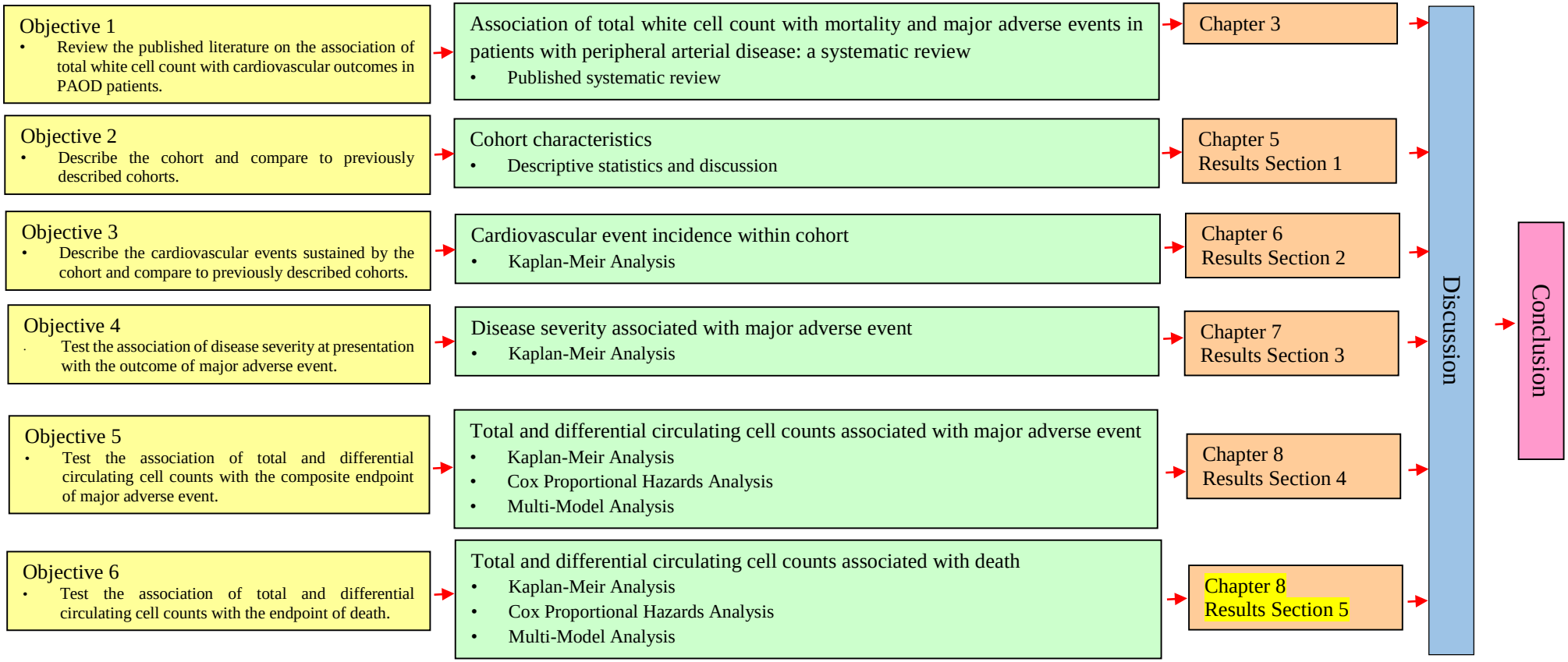

Figure 2.1: Schematic overview of thesis

2.1. Objective 1

Systematic review of the published literature on the association of total white cell count with cardiovascular outcomes in patients with peripheral arterial occlusive disease. This objective is addressed and presented in Chapter 3.[223]

2.2. Objective 2

Describe the cohort characteristics and compare to previously described cohorts with peripheral arterial occlusive disease. This objective is addressed in Chapter 5.

2.3. Objective 3

Describe the cardiovascular events sustained by the cohort and compare to previously described cohorts with peripheral arterial occlusive disease. The cardiovascular events of interest were major adverse event (composite endpoint of death, heart attack or stroke), death, heart attack, stroke, major amputation and peripheral revascularisation. This objective is addressed in Chapter 6.

2.4. Objective 4

Test the association of disease severity at presentation with the outcome of major adverse event (composite outcome of death, heart attack or stroke) in this population of patients with peripheral arterial occlusive disease.

2.4.1. Hypothesis 1

Disease severity is associated with the outcome of major adverse event (composite outcome of death, heart attack or stroke) in a population of patients with peripheral arterial occlusive disease. This hypothesis is addressed in Chapter 7.

2.5. Objective 5

Test the association of total and differential circulating cell counts with the endpoint of major adverse event (composite outcome of death, heart attack or stroke) in this population of patients with peripheral arterial occlusive disease.

2.5.1. Hypothesis 2

Baseline total and differential circulating cell counts are associated with major adverse events (composite outcome of death, heart attack or stroke) in patients with peripheral arterial occlusive disease.

The circulating cell counts investigated were total white cell count, neutrophil count, lymphocyte count, monocyte count, haemoglobin concentration and calculated neutrophil-lymphocyte ratio. This hypothesis is addressed in Chapter 8 Section 4. This hypothesis was tested using null hypothesis testing methods of Kaplan-Meir survival analysis, log rank testing and Cox proportional hazards. The novel Information-Theoretical approach of multi-model averaging was then used to compare the circulating cell counts with traditional risk factors for the outcome of major adverse event. A resultant averaged model was created to predict the outcome of major adverse event in the studied population of patients with peripheral arterial

occlusive disease using circulating cell counts, clinical disease severity, traditional risk factors, aspirin and statin use.

2.6. Objective 6

Test the association of total and differential circulating cell counts with the endpoint of death in patients with peripheral arterial occlusive disease.

2.6.1. Hypothesis 3

Baseline total and differential circulating cell counts are associated with death in patients with peripheral arterial occlusive disease.

The circulating cell counts investigated were the same as previously detailed in Hypothesis 2 however the outcome is the singular cardiovascular endpoint of death. This hypothesis is addressed in Chapter 8 Section 5. This hypothesis was testing using null hypothesis testing methods of Kaplan-Meir survival analysis, log rank testing and Cox proportional hazards. The novel Information-Theoretical approach of multi-model averaging was then used to compare the circulating cell counts with traditional risk factors for the outcome of death A resultant averaged model was created to predict the outcome of death in the studied population of patients with peripheral arterial occlusive disease using circulating cell counts, clinical disease severity, traditional risk factors, aspirin and statin use.

Each chapter in the thesis will be preceded by representation of the schematic overview of the thesis with red outline highlighting the position within the overall thesis. For example the following Chapter 3[223] is preceded by Figure 3.1 with red highlight box around the position within the overall document.

2.7. Scope of the thesis

This thesis includes a systematic review examining the available literature on the association of circulating cells and the outcomes of major adverse event and death in patients with peripheral arterial occlusive disease. A prospectively recruited cohort of 632 patients is then considered for inclusion into the study and the characteristics of the included cohort and incidence of cardiovascular events over the duration of the study are described. The total and differential cell counts are then tested for association with the composite outcome of major adverse event and the singular cardiovascular outcome of death using traditional null hypothesis testing methods and the Information-Theoretical approach of multi-model analysis. An averaged model is then developed for each outcome of major adverse event and death and methods of applying this model by the bedside or in the outpatient clinic are described. The aim of this thesis is to provide prognostic information for individual patients that may aid patient compliance with medical therapy and guide clinical decision making. Suggested directions for using the outcomes of this thesis to guide further research are also discussed.

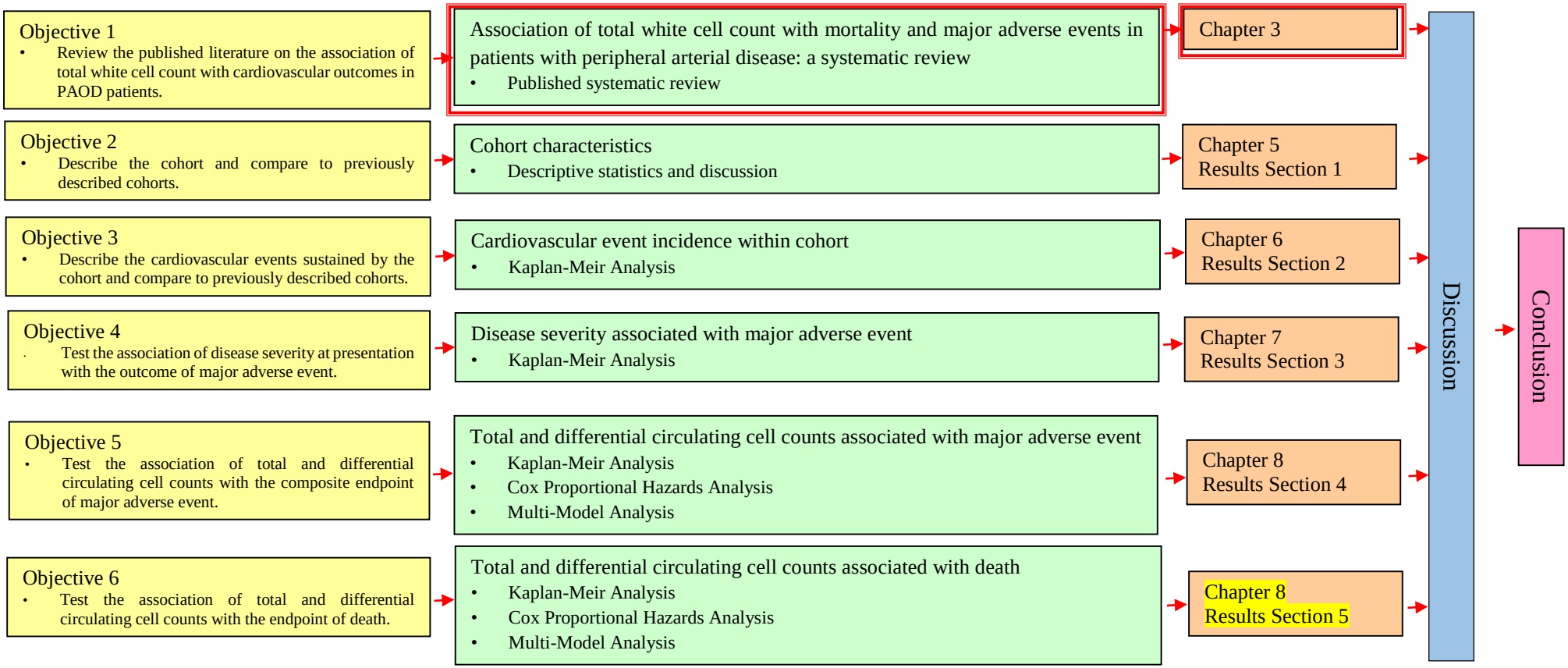

Figure 3.1: Schematic overview of thesis with red box highlighting current position within document – Association of total white cell count with mortality and major adverse events in patients with peripheral arterial occlusive disease: systematic review

3. Association of total white cell count with mortality and major adverse events in patients with peripheral arterial occlusive disease: a systematic review[223]

3.1. Abstract

Objectives: Peripheral arterial occlusive disease (PAOD) is principally caused by atherosclerosis an established inflammatory disease. Total white cell count (TWCC) is a marker of inflammation and has been associated with outcomes for patients with inflammatory diseases. The aim of this systematic review was to assess the association of TWCC with mortality and major adverse events (MAE) in PAOD patients.

Methods: Studies investigating the association of TWCC with outcome in patients with PAOD were identified by a literature search using Medline and Cochrane databases. To be eligible for inclusion studies needed to investigate the association of TWCC with mortality or a composite endpoint that included mortality in patients with PAOD. Studies were excluded when the primary focus was carotid artery disease, aortic aneurysmal disease, intracranial vascular disease, rheumatoid arthritis and treatment with chemotherapy or transplantation of stem cells. Secondary searching of reference lists and relevant reviews was performed.

Results: Ten studies including 8490 patients with PAOD met the inclusion criteria. All studies investigated more than 100 patients with four studies assessing more than 1000 patients. Study quality varied with well-established risk factors of outcome such as age, smoking, diabetes and

ankle brachial index being adjusted for inconsistently. The study populations were also disparate. Few studies reported relative risk and 95% confidence intervals for the association of TWCC with mortality or MAE. TWCC was positively and significantly associated with death alone in four of five studies investigating 3387 patients. TWCC was positively and significantly associated with MAE in five of six studies investigating a total of 6848 patients.

Conclusion: Current evidence suggests a positive association of TWCC with mortality and MAE in patients with PAOD. Further well designed prospective studies are required with high quality analysis and more complete reporting of outcomes.

Additional note: Publications revealed through replication of literature searching prior to thesis submission are included in the introduction section of this thesis.

3.2. Introduction

Peripheral arterial occlusive disease (PAOD) is one manifestation of the inflammatory disease atherosclerosis, characterized by arterial stenosis and occlusions of peripheral arteries.[12] PAOD prevalence is approximately 29% in people aged 70 years or older or those aged over 55 years with risk factors of smoking or diabetes.[5] Inflammation plays a key role in the development, progression and complications of atherosclerosis. Inflammatory processes interacting with endothelial dysfunction initiate a progressive process within the arterial wall resulting in reduction or obstruction of blood supply to end organs of the body including brain, heart, intra-abdominal organs and the tissues of the lower limbs causing morbidity and mortality.[44,46,52] Patients with PAOD have up to six times the ten year mortality of age matched controls.[19,29] The presence of either symptomatic or asymptomatic PAOD is an indicator of diffuse and significant disease in all arterial beds,[11] including coronary and cerebral arteries, and associated with a high cardiovascular event rate.[38] Predicting the risk of cardiovascular events in individuals with PAOD enables management to be tailored appropriately, for example by intensifying medical management or offering lower risk operative or endovascular management. Established predictors of outcome for patients with PAOD include age,[112] gender,[79] smoking,[28] diabetes,[95] ankle brachial index[20] and medications including antiplatelet therapy[117] and HMG-CoA reductase inhibitors (statins).[125] Markers of inflammation are well established risk predictors in patients with coronary heart disease[29,44,64] but have been relatively less well investigated in patients with PAOD.

PAOD patients have increased circulating markers of inflammation compared to the population without this disease.[53,307] Total white cell count (TWCC) assessed within a peripheral venous sample is recognized as a marker of inflammation and altered concentrations of monocytes and

neutrophils have been implicated in atherosclerosis.[46] Leucocytosis has been associated with reduced survival in patients with coronary heart disease.[99] TWCC has been shown to be a reliable predictor of outcome in patients with stable coronary heart disease[170] or acute coronary syndrome,[227,242] and after coronary artery bypass grafting (CABG)[210,211,217,250,251] or percutaneous coronary intervention (PCI).[212,215,308] TWCC also predicts mortality in other surgical[252-255,258] and cancer[309-312] patients.

It has been suggested that risk stratification models of PAOD patients should combine clinical risk factors, clinical stage of disease, a measure of PAOD severity (ABI), and a measure of systemic inflammation.[133] TWCC is routinely measured in patients with PAOD and could be added to models used to predict outcome for these patients. The independent association of TWCC with outcome in PAOD patients has however not previously been systematically examined. The aim of this systematic review was to examine the association of TWCC with mortality and the composite endpoint of major adverse events (MAE) in patients with PAOD.

3.3. Methods

Protocol: The preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines[313] and meta-analysis of observational studies in epidemiology (MOOSE) proposal[314] were followed using a standardized written protocol. Searching and study selection was conducted independently by two investigators.

Search criteria: A literature search was performed of articles published between 1st January 1946 and 1st November 2013. Databases searched included Medline (via PubMed and Ovid SP 1946 - present including in-process and non-indexed citations), and Cochrane Database of

Systematic Reviews. The search was conducted using the following terms: ("Arteriosclerosis" OR "Peripheral Arterial Disease" OR "Peripheral Vascular Disease" OR "Arterial Occlusive Disease") AND ("Leucocyte Count" OR "Leukocyte Count") with prior checking in the MeSH database to include synonyms. All subheadings were included in the searches. Publications selected for inclusion had their keywords searched manually to ensure no alternative expression or relevant keywords were unintentionally excluded. Reference lists from included publications and relevant literature reviews were also examined to increase the yield of possible studies. Studies identified in the latter way are shown in Figure 3.2 as additional records.

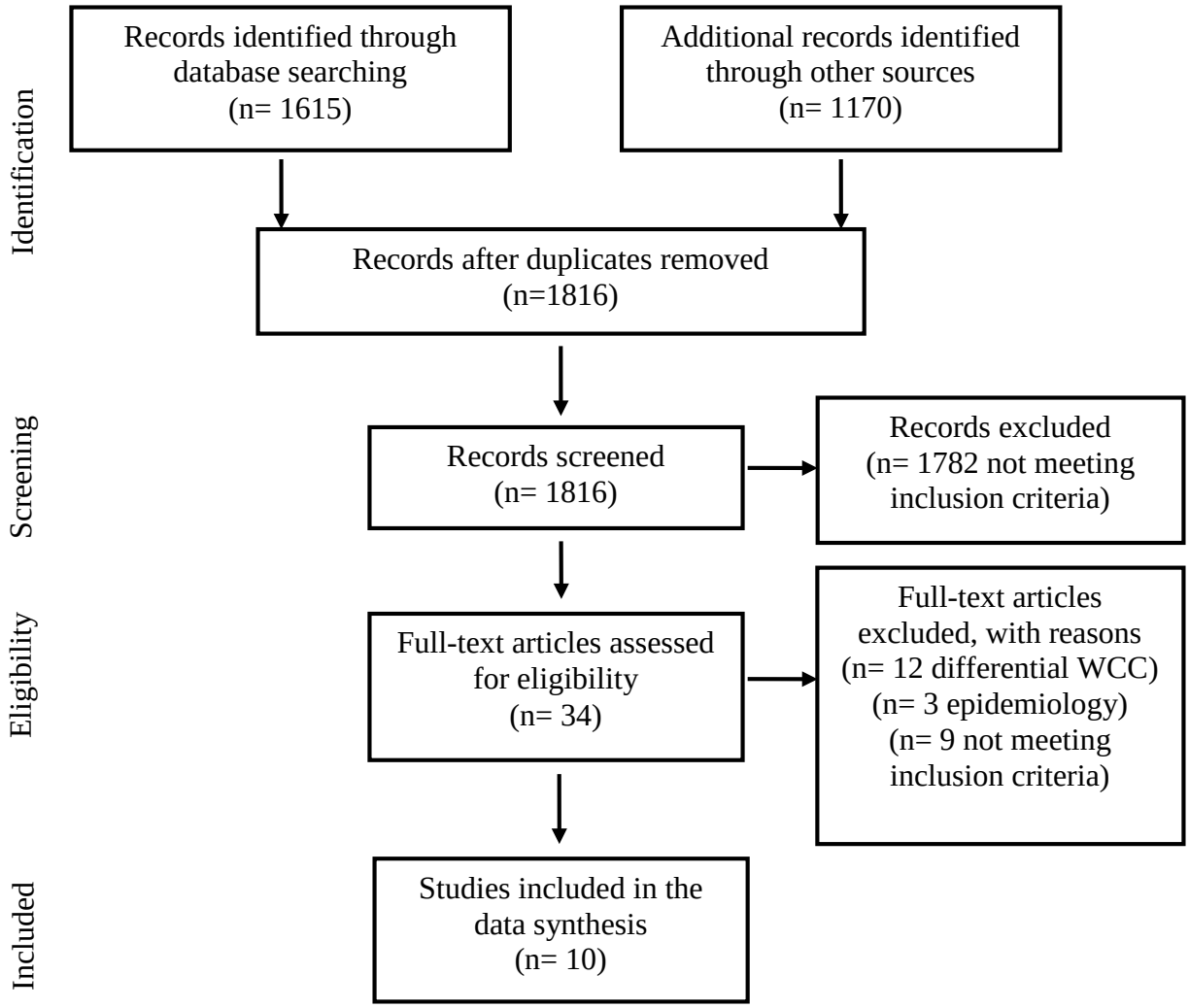

Figure 3.2: PRISMA flowchart

WCC = white cell count
n = number of participants

Eligibility criteria: For inclusion in this review studies needed to assess the association of TWCC with mortality or a composite of endpoints including death in patients with PAOD. Only publications in English were included. Studies were excluded when the primary focus was carotid artery disease, aortic aneurysmal disease, intracranial vascular disease, rheumatoid arthritis or treatment with chemotherapy or transplantation of stem cells.

Data extraction: Data was extracted from included studies by two independent investigators. Information collected included: study design; participant profile including age, gender, cardiovascular risk factors, medications and method of measuring severity of occlusive PAOD; study inclusion and exclusion criteria; and duration of follow up. The end-point definitions with type and incidence of events were recorded. The method of statistical analysis and subsequent results were also recorded. Data was standardized to include both event numbers and percentages of the relevant study population where possible. Potential sources of bias or conflict of interest were recorded.

Data analysis: Data was transcribed into a Microsoft Excel worksheet. Any discrepancies between investigators were reviewed by the author panel until a consensus was reached. No published quality assessment tool was found to assess study quality in PAOD. Therefore, a novel quality assessment tool was developed to assess the risk of bias.[315] The tool was designed to assess the inclusion of specific confounding variables relevant to PAOD within individual studies to enable comparison between studies. Risk of bias in methodology or reporting was estimated by assessing sample size, whether established risk factors for outcome in PAOD were adjusted for in analyses and follow up time (Table 3.1).

Table 3.1: Risk of bias assessment tool

Risk of Bias	Sample Size	Confounding Variables Adjusted (Univariate / multivariate)						Follow up
		Gender	Age	Smoking	Diabetes Mellitus	Medications	Ankle Brachial Index	
High	<100	Not Reported	Not Reported	Not Reported	Not Reported	Not Reported	Not Reported	<1 year
		Not statistically adjusted	Not statistically adjusted	Not statistically adjusted	Not statistically adjusted	Not statistically adjusted	Not statistically adjusted	
Medium	>100 <1000	Recorded not reported	Recorded not reported	Recorded not reported	Recorded not reported	Antiplatelet and statin reported	N/A	1-2 years
		Statistical adjustment unclear	Statistical adjustment unclear	Statistical adjustment unclear	Statistical adjustment unclear	Statistical adjustment unclear	Statistical adjustment unclear	
Low	>1000	Reported	Reported	Reported	Reported	Reported	Reported	>2 years
		Statistically adjusted	Statistically adjusted	Statistically adjusted	Statistically adjusted	Statistically adjusted	Statistically adjusted	

3.4. Results

Study selection: Searching identified a total of 688 articles in Medline and 927 in PubMed (Figure 3.2). No relevant reviews were found in the Cochrane database of systematic reviews. After combining with 1170 additional articles the 969 duplicates were removed. 1782 articles screened did not meet inclusion criteria. Thirty four full text articles were reviewed. Three studies investigating the association of TWCC with the presence of PAOD without any assessment of the incidence of morbidity or mortality of patients with PAOD were excluded (labelled epidemiology in Figure 3.2). Twelve articles were excluded for the following reasons: measurement of neutrophil-lymphocyte ratio without TWCC;[260] inclusion of TWCC but no test for the association with mortality or a composite endpoint including mortality;[152] investigation of thoracic endovascular aneurysm repair patients which are a separate subset of the PAOD population;[261] outcome measures did not include mortality or a composite endpoint including mortality[238,316-319] exclusion of patients with endpoints of clinical importance such as graft occlusion, myocardial infarction, stroke or patients requiring arteriography.[266] Ultimately ten studies were included in this systematic review.[24,49,72-74,122,222,320-322]

Study quality: The assessment of bias data for all the selected studies are shown in Table 3.2. All selected studies involved more than one hundred patients, with four studies including greater than 1000 patients.[24,49,73,320] All studies adjusted for gender in statistical analysis although adjustment for other variables was less consistent, with neither Belch et al. [12] or Violi et al. [73] reporting adjusting for age. Smoking has a well-established association with TWCC and mortality but was not adjusted for in two studies[122,320]while Arain et al. [222] reported adjusting for smoking status but did not publish details. Diabetes was not adjusted for in two included studies[320,321] despite evidence that PAOD patients with diabetes have higher rates of

mortality than PAOD patients without diabetes.[95,323] The trend for included papers to not report or adjust for medications and ABI raises the possibility of bias in the included studies. Medication adjustment was reported in only four of the ten studies,[72,74,122,222] with the remaining papers not adjusting for antiplatelet or statin use, both of which have been reported to be associated with reduced cardiovascular events in PAOD patients.[125,324] ABI, an objective measure of disease severity with previously demonstrated association with cardiovascular events,[20],[8,104,325] was not adjusted for in five[49,122,320-322] of the ten studies.[24,49,72-74,122,222,320-322] Pedrinelli et al.[322] reported adjusting for all significant variables in a step-wise regression analysis although details were not included in report. Amaranto et al.[320] adjusted for the least confounding variables (i.e. gender and age alone), while two studies, namely those reported by Arain et al.[222] and Giugliano et al.[74], adjusted for all included confounding variables. Only one study had a follow up of less than one year with follow up being only 6 months.[12] Three studies had follow up of more than 2 years.[49,74,222]

Table 3.2: Assessment of risk of bias

Risk of Bias	Sample Size	Confounding Variable Adjustment in Statistical Analysis						Follow up
		Gender	Age	Smoking	DM	Medications	ABI	
Amaranto et al.[54]	Low	Low	Low	High	Low	High	High	Medium
		Low	Low	High	High	High	High	
Arain et al.[55]	Medium	Low	Low	High	Low	Low	Low	Low
		Low	Low	Low	Low	Low	Low	
Barani et al.[56]	Medium	Low	Low	Low	Low	Low	Low	Medium
		Low	Low	High	Low	Low	High	
Belch et al.[58]	Medium	Low	High	Low	High	High	High	High
		Low	High	Low	High	High	High	
Bhutta et al.[57]	Low	Low	Low	Medium	Low	High	High	Low
		Low	Low	Low	Low	High	High	
Dormandy et al.[59]	Low	Low	Low	Low	Low	High	Low	Medium
		Low	Low	Low	Low	High	Low	
Giugliano et al.[60]	Medium	Low	Low	Low	Low	Low	Low	Low
		Low	Low	Low	Low	Low	Low	
Haumer et al.[61]	Medium	Low	Low	Low	Low	Medium*	Low	Medium
		Low	Low	Low	Low	Medium*	Low	
Pedrinelli et al.[62]	Medium	Low	Low	Low	Low	High	High	Medium
		Medium	Medium	Medium	Medium	High	High	
Violi et al.[63]	Low	Low	Low	Low	Low	High	Low	Medium
		Low	High	Low	Medium	High	Low	

* = statin alone

Characteristics of included studies: All investigations were cohort design and eight of the ten were prospective,[12,24,72-74,122,222,322] with the remaining two studies involving retrospective examination of prospectively maintained databases.[49,320] Six studies[49,72,122,222,320,322] investigated the endpoint of all-cause mortality although Dormandy and Murray[24] did not provide information on the association of TWCC and all-cause mortality so was unable to be included in this section of the analysis. Six studies used composite outcomes with varying definitions shown in Table 3.3.[24,72-74,320,321] MAE was the most common composite outcome consisting of myocardial infarction (MI), stroke and death or vascular death in four studies.[72-74,320] An amputation score used by Belch et al. [321] gave a weighted score to amputation and death. Violi et al. [73] used a composite endpoint that in addition to MAE included major amputation and excision of ischaemic viscera.

Characteristics of included participants: The ten included studies had a total of 8490 patients with PAOD. There was marked heterogeneity between studies with respect to the included participant groups (Table 3.3). Patients recruited included those undergoing major open vascular surgery[49,320] or major endovascular surgery,[320] with critical limb ischaemia,[122,321,322] with intermittent claudication,[24,73,74] and referred for lower limb investigations.[72,222] The variation in the study populations of PAOD patients was further demonstrated in the wide range of participant characteristics (Table 3.4). The inclusion of male participants varied from 50-93%.[49,122] The average age of included patients was also widely ranging varying from 63-80 years.[73,122] The prevalence of smokers ranged from 25-77%[74,122] although the definition varied across studies with Belch et al. [321] putting both current and previous smokers in the same group but Pedrinelli et al. [322] recording both current and former smokers separately. The prevalence

of hypertension varied from 35-84%,[73,320] hypercholesteremia from 47-82%[72,222] and diabetes from 25-57%.[122,222] A history of previous MI was present in only 2% of the patients in the study reported by Amaranto et al. [320] whereas 54% of the patients in the investigation of Arain et al. [222] had a history of coronary artery disease or cerebrovascular disease. The severity of PAOD as assessed by ABI was reported in six studies.[24,72-74,122,222] In the study of Dormandy et al.[24] 16% of patients had ABI <0.05 despite clinically being graded as Rutherford stage 1-3. In the study of Barani et al.[122] mean ABI was 0.31 and 28% of the non-survival group presented with gangrene, in contrast to the study of Arain et al.[222] where 53% had ABI >0.9. Renal impairment was reported in only two studies.[122,320]

Table 3.3: Study Characteristics

Author	Sample Size	Cohort Design	Inclusion Criteria	Exclusion Criteria	Primary Endpoint	Secondary Endpoint	Follow up (mo)
Amaranto et al.[320]	1773	R[a]	Major Vascular Surgery: Endovascular: CAS, T/EVAR, LES (804) Open: CEA, T/AAA, LEB (969)	'Major' elective endovascular or open intervention within 30 days of vascular index procedure, No pre-op TWCC, TWCC outside normal range	Death, MAE, TIA, Infection, Bleeding, Re-operation & amputation	Death MAE = death, stroke and MI	14[c]
Arain et al.[222]	226	P	Referral for lower-extremity vascular evaluation	ABI >1.3, not consented, lost to follow up	All-Cause Mortality	-	71 [c]
Barani et al.[122]	259	P	Admission with Critical Limb Ischaemia	No written consent	All-Cause Mortality	Vascular death	12
Belch et al.[321]	366	P	Critical Limb Ischaemia	Infection	Amputation score 1-7 [d]	-	6
Bhutta et al.[49]	1021	R[a]	Major Vascular Surgery (CEA, EVAR, Open AAA, LEB)	Fistula formation, vascular trauma, mesenteric revascularisation, thoracic aneurysm	Death within 2 years	-	24
Dormandy et al.[24]	1969	P	Claudication, ABI <0.85 in both arteries of at least one leg or re-vascularised	Platelet active drugs, β blockers, rest pain or gangrene	All-cause mortality	MAE = MI, stroke and vascular death	12
Giugliano et al.[74]	259	P	ABI<0.9 selected from patients referred to Vascular Lab (365)	ABI > 0.9; critical limb ischaemia; revascularisation <6/12; unstable angina, MI or stroke in last 3/12; decompensated heart failure; cancer; hepatic, renal or inflammatory disease. TWCC > reference range	MI, Stroke (including fatal)		30 [c]
Haumer et al.[72]	398	P	>50% stenosis on angiogram of symptomatic limb (Consecutive admissions to ward)	Acute infection unrelated to PAOD, missing data or TWCC, no follow up.	Death, MI, PCI,CABG, stroke, carotid operation,	Death alone. MAE = death, stroke and MI	20 [c]
Pedrinelli et al.[322]	108	P	Critical Limb Ischaemia undergoing angiogram	Patients on oral anticoagulants or fibrates at baseline	All-cause mortality	-	19.2 [c]
Violi et al.[73]	2111	P	Claudication, ABI <0.8 in one foot, previous amputation or revascularisation surgery	Antiplatelet, anticoagulant or NSAIDs, rest pain or ulcer, MI, stroke or surgical intervention <3/12, angina requiring CABG or PCI, liver insufficiency, renal disorders or life expectancy <2 yrs., patient withdrew, adverse events, concomitant disease, therapy refusal, antibiotics, >80 yr.	Major: Death, MI, stroke, excision of ischaemic viscera, major amputation	Minor: angina, MI, TIA, minor stroke, renal failure, HTN, vascular surgery, PTA /thrombolysis	18

CAS = Carotid artery stent
T/EVAR = Thoracic or abdominal endovascular aneurysm
LES = Lower extremity stent
CEA = Carotid endarterectomy
T/AAA = open thoracic or abdominal aortic aneurysm repair
LEB = Lower extremity bypass
TWCC = Total white cell count
MI = Myocardial infarction
TIA = Transient ischaemic attack
MAE = Major adverse event
PCI = Percutaneous coronary intervention
CABG = Coronary artery bypass grafting
ABI = Ankle brachial index
PAOD = Peripheral arterial occlusive disease
HTN = hypertension
PTA = Percutaneous transluminal angioplasty
R = Retrospective
P = Prospective
(mo) = Months
[a] = from prospectively maintained database
[b] = Non-Matched
[c] = median
[d] = Amputation score =1 no amputation to 7 hip exarticulation or death

Table 3.4: Participant characteristics

Author	Group	Cases	Male	Age	Smoking	HTN	Cholesterol	DM	Previous MI	ABI	Severity of PAOD	Renal Impairment
Amaranto et al.[320]	Endo	804	580(71%)	71.2±10.4	NR	677(83.3 %)	485(60%)	203(25.1%)	20(2.5%)	NR	NR	99(12.3%)
	Open	969	617(63%)	69.6±10.5	NR	822(84.3 %)	618 (63.4%)	251(25.7%)	19(1.9%)	NR	NR	106(10.9%)
Arain et al.[222]	Cohort	226	121 (54%)	68.4±11	137 (61%)	150(66%)	105(47%)	56(25%)	122(54%)[a]	<0.9 114 (50%)	NR	Measured NR
Barani et al.[122]	Survivor	198	99(50%)	74±10	69(35%)	141(71%)	HDL/LDL reported	100(51%)	NR	0.31±0.21	Gangrene 16(8%)	1(0.5%)
	Non Survivors	61	39 (64%)	80±10	15(25%)	40(66%)	HDL/LDL reported	35(57%)	NR	0.32±0.26	Gangrene 17(28%)	4(7%)
Belch et al.[321]	Cohort	366	221(60%)	NR[b]	244(66%)[c]	NR	NR	NR	NR	NR	NR	NR
Bhutta et al.[49]	Cohort	1021	944 (93%)	71±8.45	191[d](19%)	[d]	NR	[d]	[d e]	NR	NR	NR
Dormandy et al.[24]	Cohort	1969	1572(80%)	63.2±9.1	1396(71%)	1113 (57%)	Mean 6.5 mmol/L ±1.5	277(14%)	203 (10%)	<0.05=316 (16%), >0.85=189 (10%)	F = II	NR
Giugliano et al.[74]	Cohort	259	196(76%)	66.9±9.1	199(77%) [c]	212(82%)	200(77%)	125(48%)	115(44%)	0.69±0.18	F = II	NR
Haumer et al.[72]	NC≥ 5.8g/L	136	86(63%)	65(57-75)*	77(57%)	101(74%)	100(74%)	62(46%)	35(26%)	0.56 (0.41-0.71)*	CLI 36 (27%)	NR
	NC≤ 5.8g/L	262	147(56%)	71(60-76)*	94(36%)	196(75%)	215(82%)	95(36%)	63(24%)	0.59(0.45-0.75)*	CLI 53(20%)	NR
Pedrinelli et al.[322]	Cohort	108	78(72%)	72±10	73 (68%) [c]	88(82 %)	51(47%)	41(38%)	33(31%)[g]	0.3 (0-0.6) n=55	F = III 27 (25%) F = IV 81 (75%)	NR
Violi et al.[73]	Cohort	2111	1764(84%)	63±0.16**	718(34%)	745(35%)	NR	397(19%)	NR	<0.8 =1021(48%)	F = II	NR

Endo = Endovascular
NR = Not Reported
F= Fontaine classification system
HTN = Hypertension
DM = Diabetes Mellitus
MI = Myocardial Infarction
ABI = Ankle Brachial Index
PAOD = Peripheral Arterial Occlusive Disease
NC = Neutrophil Count
HDL = High Density Lipid
LDL = Low Density Lipid
CLI = Critical Limb Ischaemia
[a] = Coronary Artery disease OR Cerebrovascular disease
[b] = Age not reported but adjusted for in linear regression
[c] =Smokers includes current smokers and previous smokers
[d] = Recorded, Univariate regression reported
[e] = Ischaemic Heart Disease OR Cardiac Failure
[f] = Defined by statin use
[g] = Self-reported MI, angina or use of nitro-glycerine, definite MI on resting ECG, self-reported history of coronary PCI or CABG
* = Inter-Quartile Range
** = SE

Association of TWCC with death: The association of TWCC with death alone was reported in five studies including 3387 patients (Table 3.5).[49,122,222,320,322] Four studies, including 2140 patients, reported a positive association of TWCC with death after adjusting for other risk factors.[122,222,320,322] One study did not report an adjusted analysis because TWCC was not associated with death on univariate analysis.[49] In the two studies that examined major open vascular surgical procedures[49,320] neither demonstrated a significant linear association between TWCC and death but when a quadratic term was used for TWCC in the study reported by Amaranto et al.[320] a significant association was demonstrated. Odds or hazard ratios were reported in only four studies,[49,122,222,320] confidence intervals were only published in three studies[49,122,222] consequently meta-analysis was not possible. One study that did demonstrate an association[322] between TWCC and death did not publish their method of multivariate statistical analysis. Only one study[222] adjusted for all confounding variables and this study reported the most positive association of TWCC with death (risk ratio of 3.72).

Table 3.5: Association between white cell count and mortality in peripheral arterial occlusive disease patients.

Author	Population	Cases	Analysis	RR/OR/HR	95% CI	P Value	Adjusted
Amaranto et al.[320]	Major Endovascular Surgery	804	*	OR=1.82	NR	0.015	Age, gender, diabetes, CHF, MI, renal impairment, HTN, lipids, emergent procedure
	Major Open Vascular Surgery	969	*	OR=1.168	NR	0.005***	Age, gender, diabetes, CHF, MI, renal impairment, HTN, lipids, emergent procedure
Arain et al.[222]	Lower Extremity Evaluation	226	**	HR=3.37	1.56-7.27	NR	Age, gender, smoking, hypertension, CAD/CVD, serum creatinine, ABI & CRP
	Subset ABI <0.9	114	**	HR=3.72	1.38-10.01	NR	Age, gender, smoking, hypertension, CAD/CVD, serum creatinine, ABI & CRP
Barani et al.[122]	CLI	259	*	OR=1.202	1.054-1.370	0.006	NR
Bhutta et al.[49]	Major Vascular Surgery	1021	NR	OR=1.02	0.95-1.09	0.54	Univariate regression only
Pedrinelli et al.[322]	CLI	108	NR	NR	NR	<0.04	NR

RR = Relative Risk
OR = Odds ratio
HR = Hazard ratio
NR = Not reported
NS = Not significant
CLI = Critical limb ischaemia
CHF = Congestive heart failure
MI = Myocardial infarction
HTN = Hypertension
CAD = Coronary artery disease
CVD = Cerebrovascular disease
ABI = Ankle Brachial Index
*Per 1000/μL increase
**Top tertile compared to bottom tertile
*** Quadratic term for WCC
****Deceased vs alive median

Association of TWCC with MAE: The association of TWCC with MAE was assessed in six studies including 6848 patients (Table 3.6).[24,72-74,320,321] The composite outcome of MAE defined as death, MI or stroke was positively associated with TWCC in five [24,73,74,320,321] of the six included studies although definitions for inclusion in this group varied between studies. Three studies published an odds or hazard ratio[73,74,320,321] and one study relative risk[321] but only three published confidence intervals.[73,74,321] The only study reporting no association between TWCC and MAE[72] did not publish any results other than reporting no significant association. As a result of the abovementioned inconsistencies meta-analysis was not possible. Three studies reporting a positive association[73,320,321] had a high risk of bias in three or more sections of the assessment tool (Table 3.2). The remaining two positively associated studies had less marked risk of bias noted. Dormandy et al.[24] did not report adjustment for medication alone. The study reported by Giugliano et al.[74] had the lowest risk of bias of all included studies. The investigation that reported no association between TWCC and MAE adjusted for all confounding risk factors except aspirin.[72] The study reported by Haumer et al.[72] had the broadest outcome inclusion criteria, including PCI, CABG and carotid operations together with other MAE making direct comparison problematic.

Table 3.6: Association between white cell count and major adverse events in peripheral arterial occlusive disease patients

Author	Population	Cases	Endpoint	Analysis	RR/OR/HR	95% CI	P Value	Adjusted
Amaranto et al. [320]	Major Endovascular Surgery	804	MAE	*	OR 1.672	NR	0.001	NR
	Major Open Vascular Surgery	969	MAE	*	OR 0.58	NR	0.580	NR
			MAE***	*	OR 1.07	NR	0.119	NR
Belch et al. [12]	CLI	366	Amputation score[a]	$\geq 9x10^{9/L}$	RR 1.6	1.2-2.0	0.001	Gender, smoking, prostacycline Rx
Dormandy et al. [24]	Claudication	1969	MAE	NR	NR	NR	0.05	NR
Giugliano et al. [74]	Claudication	259	MAE	*	HR 1.35	1.10-1.65	<0.01	Age, gender, ABI, smoking, diabetes mellitus, hypercholesteremia, hypertension, previous MI and previous stroke
					HR 1.29	1.06-1.57	<0.02	Medications
			MAE	ROC and Bootstrap groups >7.7 $x10^9$ compared to≤ 7.7 $x10^9$	HR 3.03	1.38-6.61	0.005 Log rank	Age, gender, ABI, smoking, diabetes mellitus, hypercholesteremia, hypertension, previous MI and previous stroke
					HR 2.58	1.21 – 5.49		Medications
			MAE	ABI ≤ 0.63 and TWCC >7.7 $x10^9$ compared to ABI ≥ 0.63 and TWCC <7.7 $x10^9$	HR 5.77	2.04-16.34	0.010	NR
Haumer et al. [6172]	Inpatient Angiograms	398	MAE	**	NR	NR	0.22	NR
Violi et al. [73]	Claudication, previous amputation or bypass	2111	MAE + excision of ischaemic viscera, amputation above the ankle	Population standardised odds ratio	OR1.15	1.023 (90%) – 1.301 (09%)	0.0507	

**Top compared to bottom tertile
*** Quadratic term for WCC
RR = Relative Risk
OR = Odds ratio
HR = Hazard ratio
NR = Not reported
CLI = Critical limb ischaemia
MI = Myocardial infarction
ABI = Ankle Brachial Index
NR = Not reported
NS = Not significant
[a]Amputation Score graded 1-7 (1 no amputation to 7 hip disarticulation or death)
*Per 1000/µL increase

3.5. Discussion

There is a current deficiency in predictive markers of outcome for PAOD patients. TWCC is a recognized marker of systemic inflammation in many diseases. In the current systematic review we assessed the association of TWCC with death and MAE in PAOD patients. Overall the current studies suggest a positive but inconsistent association of TWCC with death and MAE.

Predicting the PAOD patients who will die is clinically important and prognostic indicators of risk have been proposed to include clinical risk factors, stage of disease and circulating biomarkers for these models to be accurate predictors of outcome.[133] Before these models are adopted in the clinical setting they need to be rigorously tested in studies using consistent definition of terms and thoroughness in adjusting for other known confounders.

A major finding of this review was the heterogeneity of reported studies, with great variation in the included patients and their characteristics making compilation of results challenging. This emphasizes the importance of adjusting for established confounding variables and fully reporting the results of adjusted analyses. Without this information a meta-analysis of collated studies was not possible. Whilst adjustment for some confounders was consistent between studies with all studies adjusting for gender, and all studies except those reported by Belch et al.[321] and Violi et al.[73] adjusting for age, the known confounders of medication and ABI were poorly adjusted for in the selected studies. Pedrinelli et al.[322] reported the confounders of gender, age, smoking and diabetes although it was unclear whether these variables were adjusted for in their statistical analysis. The only selected study to adjust for all confounders in the assessment tool was Arain et al.[222] who reported the strongest association of TWCC with

death. Giugliano et al.[74] was the highest quality study with the lowest assessed risk of bias, and reported the strongest positive association of TWCC with MAE.

Variation in the definition of MAE was another key finding. The endpoint of death, stroke and MI (MAE) (+/- CABG, +/- PCI) was used in three studies but in one study death from causes other than MI and stroke was not included in MAE[74]. Some investigators included re-intervention such as revascularization with bypass or endovascular surgery or major amputation with MAE. An agreed international definition for MAE in PAOD patients would assist literature review and comparison of reported series.

There is evidence that TWCC may better predict outcome in endovascular interventions and lower limb operations than when combining all major vascular operations including aortic and carotid surgery. Two studies investigating patients undergoing angiogram or major endovascular interventions were able to demonstrate an association between TWCC and outcome on both univariate and multivariate analyses.[320,322] A similar result was seen in a study that focused on lower limb bypass[316] although the sample size was small and the outcome was MI in isolation rather than a composite outcome thus not meeting inclusion criteria for this review. TWCC was not linearly associated with mortality of patients undergoing major open vascular surgery in two large groups,[49,320] though when a quadratic term was used for TWCC[320] a significant association with death was found, raising the possibility of a more complex relationship in these patients which will need to be considered in statistical analyses in the future. There are many potential confounders in open operations for aortic and carotid disease and in this patient group it may be more appropriate to examine these interventions separately in the future.

The timing of blood sampling in relation to surgical intervention was variable in the included studies. In one study the most recent TWCC prior to the procedure[320] was used, whilst in some investigations timing was not specified. Both Violi, et al.[73] and Dormandy, et al.[24] included patients that previously had arterial reconstructive surgery without reporting the timing of this surgery relative to study entry. Ulceration, tissue loss and gangrene of the lower limb are all potential confounders of TWCC in patients with PAOD. None of the studies focusing on critical limb ischaemia reported differentiating rest pain from gangrene.[72,122,321] None of the included studies adjusted for antibiotic use and only in the investigation reported by Violi et al.[73] were patients on antibiotics excluded.

The limitations of this review include the small number of studies identified and the heterogeneous nature of populations studied and endpoint definitions. TWCC is the most commonly measured inflammatory marker that has been associated with the clinical outcomes of atherosclerotic disease, although its use for risk stratification remains unproven in the PAOD population. It has been suggested that risk factor modification and targeted medical therapy in the high risk population would be of benefit;[12,135,326] and that prognostic modelling that identifies high risk patients may increase patient compliance to lifestyle change and pharmacological therapy. The relationship between TWCC and specific outcomes within defined subgroups of the PAOD population based on stage of disease requires further study before it is able to be used in modelling to predict outcome. Further analysis of differential WCC should be undertaken in the same manner, to assess its value in determining high risk patients for targeted interventions. Future studies should also standardise reporting including

timing of blood sampling, infection or tissue loss in the limb, antibiotic usage at the time of sampling and the timing and method of revascularisation in relation to sampling.

In conclusion this systematic review demonstrated a positive but inconsistent association of TWCC with death and MAE in patients with PAOD. If TWCC is associated with death and MAE in higher quality large studies as a readily obtainable marker of inflammation it may be easily integrated into predictive models of outcome for PAOD patients.

4. Study design and methodology

This chapter will describe selection of the study participants with inclusion and exclusion criteria. Data collection and retrospective data validation that was undertaken to ensure completeness of recorded cardiovascular events and potential confounding factors will be explained. Definitions and methods of recording cardiovascular endpoints and confounders will then be addressed in detail. The procedures section of this chapter will model the individual patient trajectory through the study, in particular selection of the most appropriate "study baseline bloods". Statistical analysis that was employed to test the hypotheses will conclude the chapter, including the *a priori* power calculation that was performed to ensure an adequate sample size was recruited.

4.1. Participants

This study was part of a larger prospective cohort study of patients which included patients with known peripheral arterial occlusive disease, aortic aneurysm and/or carotid artery disease. Ethical approval for this study was granted by James Cook University Human Ethics Committee ethics approval number H2196 (Appendix A), the Townsville Health Services District ethics committee Protocol 61/05 (Appendix B) and the Mater Hospital Townsville ethics committee (Appendix C). Prospective recruitment has occurred since 2002 from The Townsville Hospital and Townsville Mater Hospital during vascular surgical outpatient clinics or upon admission to hospital to undergo a vascular interventional procedure. All patients included in this study signed an informed consent form (Appendix D or Appendix F) after being given an information sheet (Appendix E or Appendix G). Patients were followed until death, discharge from clinic or the conclusion of data collection for this study (1/12/2014).

Enrolment in the study was voluntary and did not affect the clinical management of the patients in any way.

Of the 1439 patients enrolled in the larger cohort study who were recruited from the above sites before November 1 2014, 632 patients were initially selected as meeting the inclusion criteria (3.1.1 page 95) and not the exclusion criteria (Section 3.1.2 page 96) and underwent chart review and data collection. Power calculations are shown in Section 3.3.3 (page 127) which demonstrated this to be an adequate sample size.

4.1.1. Inclusion criteria

Inclusion criteria were chosen to broadly select all recruited patients with symptomatic peripheral arterial occlusive disease.

- Consecutive patients recruited into the "The role of differences in circulating factors in the pathogenesis of vascular disease" study who provided written consent.
- Patients recruited from The Townsville Hospital, Townsville Mater Hospital Pimlico and Townsville Mater Hospital Hyde Park.
- Symptomatic peripheral arterial occlusive disease – defined as having features consistent with the disease on clinical history or examination by a vascular surgical consultant.
- Pre-operative or "study baseline bloods" available for analysis. "Study baseline bloods" were defined as blood sampling: either at recruitment when well with no signs of infection or current antibiotic treatment; or greater than one month post interventional procedure or major adverse event and the absence of active infection,

antibiotic treatment or other exclusion criteria (see also “Inclusion with caution” Section 3.2.3 page 98).

- Minimum follow up of one month after admission to the study or interventional procedure. Patients were included if death occurred in this period.
- Ankle Brachial Index (ABI) is a measure of severity of peripheral arterial occlusive disease and is calculated by the Doppler systolic blood pressure at the ankle divided by the brachial systolic blood pressure. Elevated Ankle Brachial Index >1.4 results from incompressible arteries of the lower limb and has been an exclusion criteria in previous studies investigating TWCC and outcome of peripheral arterial occlusive disease.[74,222] However, these patients have a documented risk of critical limb ischaemia and raised all-cause mortality[20,116,325,327] and form an important subset of peripheral arterial occlusive disease patients therefore these patients were included in this study.

4.1.2. Exclusion criteria

- Aneurysmal disease requiring intervention – either open surgical or endovascular management.
    - Although occlusive and aneurysmal arterial disease share some common risk factors, similar pathophysiology and indeed common circulating markers of inflammation, there are some obvious discrepancies among risk factors, genetic determinants and histological disease progression.[328] The confounding effect of two similar inflammatory processes running in parallel would have been difficult to control for with the chosen statistical analysis. Therefore patients with

aneurysmal disease that was significant enough to require intervention during the course of the study were excluded.

  - Patients with small asymptomatic abdominal aortic aneurysms <5cm maximal aortic diameter and small peripheral aneurysms that did not require intervention over the course of the study were included.

- No pre-operative or "study baseline" bloods available.
- Total white cell count (TWCC) >15 $x10^9$ cells/L (reference range 3.5-11.0 $x10^9$ cells/L)
  - TWCC<15 $x10^9$ cells/L includes >99% of the non-oncology adult patients at our testing centres and values outside this range raise the possibility of confounding variables elevating the white cell count; for example subclinical infection.
  - Low TWCC <3.5 $x10^9$ cells/L were included as U or J shaped relationships between TWCC and cardiovascular endpoints have been previously reported.[89,201]
- Malignancy
- Haematological disorders
- Known cause for leucocytosis
- Renal failure requiring dialysis
  - Inflammatory markers are a predictor of outcome in chronic renal failure, however the causes of this inflammation appear different to the peripheral arterial occlusive disease population with separate risk factors, inflammatory signals associated with dialysis, decreased renal function, volume overload and inter-current clinical events all playing a contributing role.[329]
- Chart not available for review and validation of collected data.

- This excluded patients recruited from other hospitals and they were not included in this study.

- Immune modifying medication including steroids, clozapine, immune-suppressants (including but not limited to: Sirolimus, Tacrolimus, Cyclosporine, Mycophenolate Mofetil)
- Upper limb amputation
- Lower limb amputation during the course of study for trauma or tumour.
    - Trauma causes activation of systemic inflammatory immune response which is further augmented by the "second hit" of an amputation operation and can lead to systemic inflammatory response syndrome, the interactions and consequences of which are detailed elsewhere.[330] Interpretation of inflammatory markers in this setting and their correlation with mortality is outside the scope of this study.
    - Inflammation has a role in tumour establishment and progression[331] and this process will confound inflammatory marker levels. These patients were also excluded under 'malignancy'.

4.1.3. Inclusion with caution

- Active infection at time of recruitment
    - Active infection including infection of ischaemic ulcers is an important confounder because elevation of white cell count associated with infection would be indistinguishable from elevation due to inflammatory or ischaemic processes alone.
    - Infection was defined as wound culture positive for pathogens, documentation of clinician suspected infection or antibiotic prescription.

- These patients were eligible for inclusion if later blood tests were available after the clinical resolution of infection and cessation of antibiotic therapy, a minimum of four weeks post-operatively and prior to any cardiovascular endpoint

4.1.4. Data Collection and Validation

Patients were prospectively recruited from the outpatient departments or when admitted to hospital to undergo a vascular surgical procedure. A Microsoft Access (2010/2013) front end was used to access a SQL (structured query language) database server (R2, 2008) that was established to collect clinical information over time, enabling retrospective analysis of the progression of the disease process. The collected data for this study included: clinical history of presenting condition and risk factors for vascular disease; medications including total daily dose; previous cardiovascular events; results from blood analysis including biochemistry and full blood counts. Other variables and endpoints were also recorded as part of the research process although this data was not intended for use in this study but other studies underway within the larger "The role of differences in circulating factors in the pathogenesis of vascular disease" study being conducted from the same database. The data collected but not intended for use in this study included additional medical history, additional blood results including electrolytes, liver function tests, coagulation profile, lipid profile, HbA1C (measure of glycated haemoglobin – specifically the beta-N-1-deoxy fructosyl component of haemoglobin), CRP and homocysteine where available. Details of all vascular and cardiac admissions and interventions as well as all medications and dosage were recorded although were not used in this study.

The principal investigator reviewed all of the medical records of the recruited patients. All chart reviews were conducted by the author with consistent method, over the briefest time period possible. Thus inter-rater error was eliminated through the use of a single investigator and intra-rater error was reduced through consistency of method. Paper and electronic patient records were reviewed for details of every inpatient hospitalisation, outpatient visit, laboratory and pathology results, relevant correspondence and notification of death. AUSLAB (Queensland Health Clinical and Scientific Information System), Sullivan and Nicolaides Pathology and Queensland Medical Laboratories data systems were reviewed in the collection of blood analysis results. All laboratories adhered to the WHO quality assurances in haematology guidelines.[193] Validity of chart reviews can be enhanced by decreasing the number of reviewers, using consistency of method and reporting inter-rater reliability (eliminated in this study by single investigator data collection).[332] Patients were continually recruited prospectively and patients were followed in outpatient clinics to collect outcome data when being reviewed as part of their ongoing clinical care by vascular surgical consultants. Treatment of patients was not altered in any way by inclusion or exclusion from this study.

Archived data in the database is owned, controlled, and is continually edited by other investigators with separate research questions. Therefore all data was extracted from the database on completion of data collection at a single point in time. The extracted data spreadsheet was then rechecked against the database and errors from extraction were corrected for each patient in the study. Where there was concern relating to extracted data, patient medical records and electronic records were used to confirm correct results.

All information extracted from medical charts can have its validity questioned, dependent on the patient reporting and the doctor recording, chart reviews can have variable results, largely depending on the history of interest with concordance less likely in asymptomatic conditions.[333,334] This is important to acknowledge as the risk factors of peripheral arterial occlusive disease that were required to be adjusted for in statistical analysis are often asymptomatic. Subsequently the collection of this data did not come from the record of the patients self-report alone, instead efforts were taken with data collection to collect corroborating evidence from other sources both in the patient chart and electronically to minimise the effects of patient recall or single doctor recording.

Whilst every effort was made to minimise missing data, due to the nature of retrospective data validation there were some incomplete data sets which were excluded. The most common missing information was no “study baseline bloods” in 27 patients and <1 month follow up in 18 patients. Only patients that had complete data sets were included in the current study.

4.2. Outcome measures and variables

All patient information was entered through a Microsoft Access front end and stored in a SQL (structured query language) database developed for this purpose on a secure James Cook University server. All values, outcome measures and variables were validated retrospectively from multiple sources by the principal investigator to eliminate inaccuracy. The outcome measures and confounding variable definitions and reasons for inclusion in the study are presented followed by description of the method used for data collection.

4.2.1. Outcome Measures

4.2.1.1. Primary Outcome – composite of cardiovascular events

Major Adverse Event (MAE): was defined as the first event in the composite outcome of death, heart attack or stroke. Each of these are defined individually below.

4.2.1.2. Secondary Outcomes – individual cardiovascular events

Death by cause: cessation of life with cause recorded when available. Death certificates are not readily available for research purposes in the state of Queensland. The recorded arrival of the patients' body at a Queensland Health mortuary was the most common method of confirming death and autopsy to confirm cause of death when performed. In other cases collateral was sought from the patients general practitioner or treating physician to confirm date and cause if known.

Heart attack: heart muscle cell death diagnosed with evidence of myocardial necrosis consistent with ischaemia according to the universal definition of myocardial infarction (heart attack):[335]

- Cardiac biomarker (Troponin I or T, creatinine kinase –myoglobin fraction) rise above the 99th percentile.
- *And* at least one of the following:
  - Symptoms of cardiac ischaemia – chest pain or angina equivalent (arm, neck, jaw, back or epigastric pain) lasting for more than 20 minutes.
  - ECG (electrocardiogram) changes indicative of new ischaemia:
    - New ST segment change or new left bundle branch block.
    - Development of pathological Q waves on ECG.
  - Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.

- Does not include all percutaneous coronary intervention unless accompanied by peri-procedural myocardial necrosis meeting above criteria with the level of biomarker sensitivity raised to 3 x 99th percentile by convention.[335]
- Does not include all coronary artery bypass graft operations unless peri-procedural evidence of myocardial ischaemia with the conventional range 5 x 99th percentile.[335]
- If leading to death, death was the secondary outcome rather than heart attack.

Stroke: damage to function of brain cells due to either ischaemia or haemorrhage.

- Defined by sudden onset focal or global demonstrable neurological defect lasting longer than 24 hours.
- If leading to death, death was the secondary outcome rather than stroke.

Major amputation: below knee, above knee and hindquarter amputations were defined as major amputations. Digital amputation and trans-tarsal amputation were considered minor amputations. Major amputation was included as it is a clinically relevant non-fatal outcome for patients with peripheral arterial occlusive disease with considerable morbidity.

All other cardiovascular and vascular events including admissions to hospital were recorded in the database for the patients recruited to this study. Further outcomes were not intended for analysis within this study and were therefore not included as secondary outcomes.

4.2.1.3. Method of recording cardiovascular outcomes

In consultation with the director of the Department of Vascular and Endovascular Surgery unit at The Townsville Hospital, senior research nurse and database manager, the hierarchical

recording of all cardiovascular events was reviewed and upgraded prior to the commencement of data collection. The purpose of this upgrade was to improve the accuracy of the database records and enable subgroup analysis by specific cardiovascular event. The hierarchical redesign was done to clearly record differentiated cardiovascular events and did allow for collection of more cardiovascular event detail than was analysed by this study but will be referenced with greater ease during future research on this patient group.

4.2.2. Variables

Full blood counts which include total and differential white cell count and haemoglobin concentration were measured by standardised means by Pathology Queensland Clinical and State-wide Service (the pathology service for Queensland Health), Queensland Medical Laboratories (QML) Pathology and Sullivan and Nicolaides Pathology Laboratories only. Total white cell count (TWCC) has been demonstrated to have diurnal[336] and seasonal variation[337] so date and time of blood collection was recorded and analysed to minimise unrecognised systematic error from data collection. Preliminary analysis of the time of blood sampling in this study (with linear regression and best fit residual calculations) showed no correlation to cell count values or cardiovascular events and is therefore not controlled for as a variable or presented in the results. An overview of the precedence for inclusion of each of the individual circulating cell parameters for their association with cardiovascular events is presented in Chapter 1.

Activation of white blood cells by smoking and hypertension[338] or ischaemia increases their adhesion to endothelium[158] and makes them much less deformable (~2000 times) than red blood cells[321,339] possibly contributing to the small vessel plugging[340] and the cascade that

follows to produce ischaemic events.[177] Activated white blood cells may be released from ischaemic tissue and cause adverse effects in remote organs.[230,231,341] Whilst measurement of activation of white blood cells was outside the scope of the current study, mobilisation of white blood cells from the marginalised pool is an important potential contributor to an elevated circulating white cell count in this population.

4.2.3. Confounding Variables

Data was collected on variables other than circulating cell counts that may either predispose or protect patients from cardiovascular events. These variables have been grouped into risk factors and medications. Disease severity at presentation is also a confounding variable and definitions and rationale for inclusion will be presented following risk factors and medications.

4.2.3.1. Patient Risk Factors

Although commonly referred to as risk factors for peripheral arterial occlusive disease in the clinical setting, many of the confounding variables below have only been demonstrated to have an association with peripheral arterial occlusive disease. Smoking and hypercholesterolemia are the only two true risk factors; having been shown in prospective controlled studies that altering the factor affects the course of the disease.[28] The other confounding variables presented in Table 4.1 have been shown to be associated with mortality and cardiovascular events and therefore were adjusted for in statistical analysis.

Table 4.1: Risk factors and precedent for association with cardiovascular events

Confounding variables - risk factors	Definition for data collection	Precedent of association with cardiovascular events

Age	Years, Days (calculated from date of birth)	Increase in both incidence and prevalence of PAOD with increasing age.[28] Mortality is also a function of increasing age.[78]
Sex	Male/Female	Prevalence of both symptomatic and asymptomatic PAOD is higher in men than in women with the ratio increasing in more severe stages of the disease.[1,2,80] Some studies report a higher incidence of women with critical limb ischaemia.[9] Cardiovascular mortality in both asymptomatic and symptomatic PAOD is significantly higher in men.[79]
Diabetes Mellitus	Diagnosis of diabetes or on treatment for diabetes	Mortality is higher in PAOD patients with diabetes mellitus.[95] Patients with critical limb ischaemia have higher rates of major amputation and mortality if diabetic.[97] HbA1C and diabetic medications including dose were recorded in the database where available but this data was not available for all patients and was not intended for analysis in this study.
Smoking	Never, Current or Ex-smoker (>30days) Cigarettes smoked per day Years smoked	The number of cigarettes smoked is associated with presence of PAOD, disease severity, risk of amputation, graft occlusion and mortality.[28] Patient report of their smoking was used for this study as this has been previously established to be a valid measure.[342] Objective measure of smoking habit such as serum cotinine, thiocyanate or CO exhale are more accurate in confirming smoking habit in certain populations[343] although was not available to be applied across this entire cohort.
Ischaemic Heart Disease	Diagnosis of ischaemic heart disease or on treatment for ischaemic heart disease	Ischaemic heart disease is a predictor of short and long term mortality in vascular patients undergoing revascularisation or aneurysm repair.[105]
Stroke	Medical diagnosis of stroke	A combination of stroke or transient ischaemic attack (TIA) was one of the identified risk factors that predicted mortality at 5 and 10 years in a review of mortality in 2642 consecutive PAOD patients.[106] Touze et al.[107] quantified the risk of heart attack and non-stroke vascular mortality to ~2% per year following stroke or TIA.
Hypertension	Diagnosis of hypertension or on treatment for hypertension	Hypertension increases the risk of PAOD[28] and PAOD increases the risk of mortality,[24,104] but whether hypertension is an independent risk factor within the PAOD population for mortality remains unclear.

PAOD = Peripheral arterial occlusive disease; CLI = Critical limb ischaemia; TIA = Transient ischaemic attack

4.2.3.2. Medications

All participants in this study had their medications recorded at entry into the study and reviewed at subsequent outpatient appointments or admission to hospital. All medications were recorded with total daily dosage. Vascular patients are commonly on multiple medications and an overview by description of the total medication usage of the cohort will be presented in Chapter 5, but as all medications were not considered significant confounders they will not be presented

here. The two medications that were adjusted for as potential confounding variables are described in Table 4.2 with justification for inclusion as confounding variables.

Table 4.2: Medications and precedent for association with cardiovascular events

Confounding Variables Medications	Precedent for medication association with cardiovascular events
Aspirin	The Antithrombotic Trialists Collaboration analysed 287 randomised trials including more than 135000 patients and reported that the odds of a vascular event (vascular death, non-fatal heart attack or nonfatal stroke) were reduced by 22% in high risk patients receiving antiplatelet therapy.[117] Consistent in the subset of patients with diagnosed PAOD.[117]
Statins	The JUPITER trial (Justification for the use of statins in Prevention: an intervention trial evaluating Rosuvastatin use) in a non-PAOD population demonstrated a 44% reduction in all-cause and cardiovascular morality and a concomitant reduction in CRP levels.[119]. The Heart Protection Study demonstrated a 22% risk reduction for first major vascular event in PAOD patients allocated to Simvastatin.[120] Short term statin use has been shown to reduce major adverse events within six months of vascular surgery.[123,124] Shillinger et al.[125] demonstrating that PAOD patients receiving Statin therapy for a minimum of 4 weeks prior to peripheral endovascular intervention had significantly improved survival and event free survival rates. Further analysis showed that the patients with the higher markers of inflammation benefited the most from statin therapy.[125] Statin use has also been associated with improved graft patency and limb survival[127] and has been shown to have anti-inflammatory effects.[128]

PAOD = Peripheral arterial occlusive disease; CRP = C reactive protein; JUPITER = Justification for the use of statins in prevention: an intervention trial evaluating Rosuvastatin.

4.2.3.3. Disease severity at presentation

The severity of an individual patient's disease was assessed by a vascular surgical consultant on recruitment to the study with both clinical history and physical examination. The disease severity was classified as either asymptomatic (excluded from this study), intermittent claudication, rest pain, tissue loss or acute lower limb ischaemia, a combination of both the Rutherford[344] and Fontaine[345] classification systems for peripheral arterial occlusive disease as seen in Appendix I.

Patients with intermittent claudication are expected to have an annual major adverse event rate of 5%-7% approximately 2.5 times higher than age matched controls independent of risk factors.[28] Patients with rest pain or tissue loss have a ~20% mortality in the first year after presentation.[28] The division of critical limb ischaemia into rest pain and tissue loss groups has been suggested previously as these two groups have appreciably different natural histories.[26] The short term mortality of patients presenting with acute lower limb ischaemia is 15-20%, with long term mortality risk after resolution of the acute event reverting to that of the claudication group or rest pain and tissue loss group depending on the outcome of the acute event.[28] For this reason, the patients with acute limb ischaemia at recruitment to the study are presented and analysed separately throughout the results chapters.

4.3. Procedures

Study participants had all risk factors, medications and any history of cardiovascular event recorded at point of entry into the study. At each outpatient appointment any subsequent cardiovascular events or significant vascular events were recorded along with review and recording of the patient risk factors and medication use. Measures of circulating differential cell count were recorded at study entry but not at subsequent cardiovascular event or significant vascular event unless this was the entry point to the study. Four hypothetical patients are now presented as examples of individual participant progress through the study, the data collection points and the appropriate selection of “study baseline bloods”.

4.3.1. Example 1: “Hypothetical Patient A”

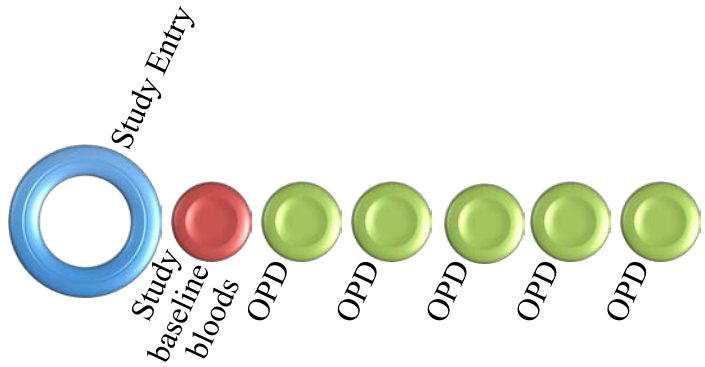

Figure 4.1: Progress of Hypothetical Patient A through the study.
(OPD, outpatient department)

A pictorial representation of the course of the individual participant “Hypothetical Patient A” through this study is shown in Figure 4.1. Hypothetical Patient A was recruited into the study after meeting the inclusion criteria with demonstrable symptomatic peripheral arterial occlusive disease (represented in blue Figure 4.1). Hypothetical Patient A has “study baseline bloods” (represented in red Figure 4.1) soon after recruitment to the study. This patient is managed non-operatively through out-patient department (OPD) appointments (represented in green Figure 4.1). The patient’s risk factors and medications were reviewed and recorded at each outpatient visit. Medications may have been introduced to reduce the risk of future cardiovascular events and these were recorded at each visit. Hypothetical Patient A did not sustain any cardiovascular events and was discharged from follow up (from the study) at the conclusion of data collection for the study.

4.3.2. Example 2: "Hypothetical Patient B"

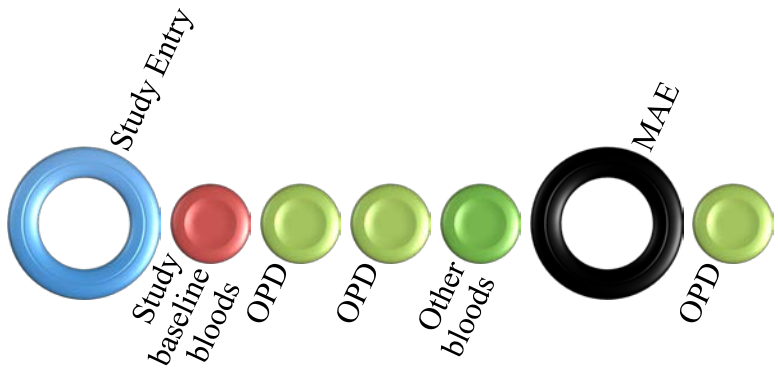

Figure 4.2: Progress of Hypothetical Patient B through the study.
OPD = Outpatient Department appointment, MAE = Major adverse event (death, heart attack or stroke)

Figure 4.2 shows 'Hypothetical Patient B' who was recruited (represented in blue in Figure 4.2) from the outpatient department (OPD) with symptomatic peripheral arterial occlusive disease and has study baseline bloods (represented in red in Figure 4.2) at the time of recruitment. While being followed up as an outpatient having all risk factors and medications reviewed at each visit, Hypothetical Patient B experiences a non-fatal major adverse event (MAE) (represented in black in Figure 4.2). The type of major adverse event (death, heart attack, stroke or major amputation) and time from study entry were both retrospectively entered into the database. The bloods taken immediately prior to or at the time of the major adverse event ("Other bloods" represented in orange, Figure 4.2) were not used in this study. At the time of any cardiovascular event (including major adverse event) risk factors and medication use were again reviewed. Hypothetical Patient B did not die as a result of the sustained major adverse event and was subsequently reviewed in the outpatient department and was discharged from follow up (from the study) at the conclusion of the study having sustained an MAE but not death.

4.3.3. Example 3: "Hypothetical Patient C"

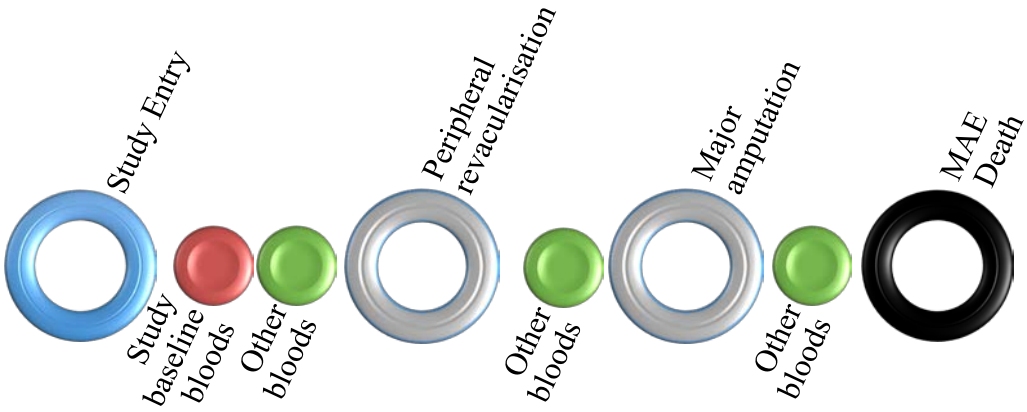

Figure 4.3: Progress of Hypothetical Patient C through the study

MAE = Major adverse event (death, heart attack or stroke)

Hypothetical Patient C is shown in Figure 4.3, pictorially representing the progressive nature of peripheral arterial occlusive disease. "Patient C" requires peripheral revascularisation (represented as green in Figure 4.3), this event was recorded in the database and risk factors and medication use were reviewed. Patient C later requires major amputation (both significant vascular events represented as green in Figure 4.3) with the event recorded in the database and risk factors and medication use again reviewed. The time from recruitment to major amputation was retrospectively calculated. For this study, only the bloods taken at, or shortly after, study entry and not associated with a vascular intervention or major adverse event will serve as the "study baseline bloods" (represented as red in Figure 4.3). Bloods taken at the time of peripheral revascularisation, major amputation and prior to the fatal event ("Other bloods" represented in orange, Figure 4.3) were not recorded. Time from study entry to major adverse event and time from study entry to death would be the same for this patient as the first major adverse event was death.

4.3.4. Example 4: “Hypothetical Patient D”

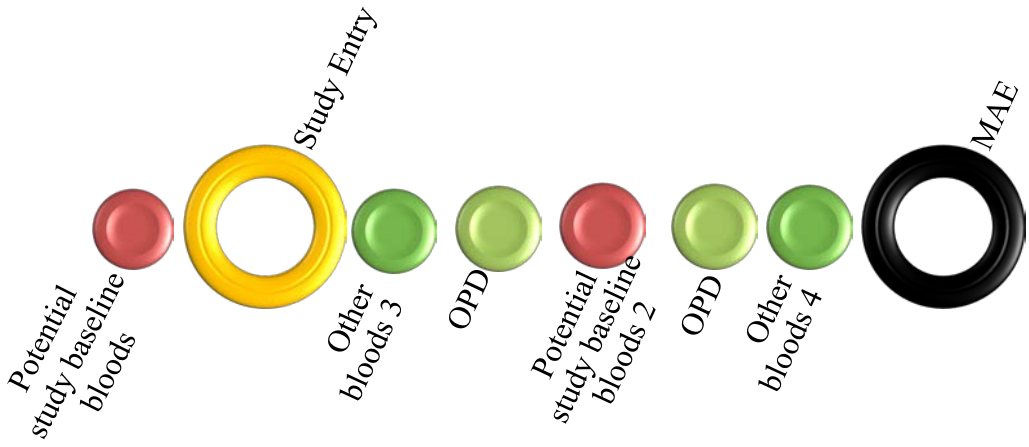

Figure 4.4: Progress of Hypothetical Patient D through the study

(OPD = Outpatient Department appointment, MAE = Major adverse event (death, heart attack or stroke)

Hypothetical "Patient D” is recruited to the study as the result of a non-fatal significant vascular event (vascular admission to hospital or vascular intervention). Pre-operative bloods for this patient (“Potential study baseline bloods”, represented in red, Figure 4.4) were used for “study baseline bloods” unless active infection was present or the patient was on antibiotic therapy. For example if the significant vascular event was revascularisation for tissue loss or gangrene with concomitant infection, or minor or major amputation with infection of tissue recorded, then the “study baseline bloods” were a minimum of 4 weeks post-operatively if infection had resolved and the patient was no longer on antibiotic therapy (“Potential study baseline bloods 2”, also represented in red, Figure 4.4). Bloods taken in the post-operative period (“Other bloods 3”, represented in orange, Figure 4.4) were not recorded or analysed due to the inability to control for post-operative change in inflammatory markers. “Other Bloods 4” (represented in orange, Figure 4.4) were not recorded or analysed due to the close association with subsequent major adverse event.

4.4. Data Analysis

4.4.1. Kaplan-Meier log rank survival analysis

Kaplan-Meier analysis is used throughout all results chapters to present and compare the freedom from endpoint (survival) using TIBCO Spotfire S+ 8.2 for Windows 2010 (TIBCO Software Inc. Boston, USA). The Kaplan-Meier survival analysis allows comparison of populations within the study, taking account of the patients discharged from follow up without event.

The survival rate is expressed as the Kaplan-Meier survivor function (S) is seen in Equation 1:

$$S(t) = \frac{number\ of\ individuals\ surviving\ longer\ than\ t}{total\ number\ of\ individuals\ studied}$$

Equation 1: Survivor rate for Kaplan-Meier analysis.

Where S = Kaplan-Meier survival function,
t = time.

The product limit method of Kaplan-Meier[346] is used to estimate S (Equation 2):

$$\widehat{S}(t) = \prod_{t_i \leq r} \left(1 - \frac{d_i}{n_i}\right)$$

Equation 2: Product limit method of Kaplan-Meier.

Where $\hat{S}$ = an estimate of the survival function,
t_i = duration of study at point i,
d_i = number of events up to point i,
n_i = number of individuals at risk just prior to t_i

S is based upon the probability that an individual survives at the end of a time interval. If a subject is last followed up at time t_i and then leaves the study for any reason (e.g. is discharged from follow up) t_i is counted as their censorship time. Censoring indicates that the period of observation was terminated prior to the patient experiencing the outcome of interest and occurs

for two reasons in this study, the patient was discharged from follow up or the study data collection period ended (and the patient was discharged from study follow up).

Survival is not the absence of death but the absence of the endpoint in question and the statistical term of survival used in Kaplan-Meier analysis has the potential to become confusing when discussing some terminal and some non-terminal outcomes in relation to cardiovascular events. For this reason the survival function in this study will refer to the freedom from death, freedom from heart attack, freedom from stroke, freedom from peripheral revascularisation and freedom from the composite outcome of major adverse events as specified. For example, the survival rate for the endpoint heart attack is referred to as freedom from heart attack at time point t_i.

Samples of survival times are frequently highly skewed, therefore although both the median and the mean are presented throughout the results chapters, the median is generally considered a better measure of central location than the mean. The median survival time is calculated as the smallest survival time for which the survivor function is less than or equal to 0.5. In some of the following data sets the survivor function is always greater than 0.5, therefore the median survival time was not able to be calculated and is expressed as n/a. A confidence interval for the median survival time is constructed using a robust nonparametric method.[347] This confidence interval is not applicable if the median survival time is not calculable and is expressed as n/a. With skewed survival data, calculation of the upper confidence interval using the Bookmeyer-Crowley[347] method may result in an answer of infinity due to a need to divide by 0 in the calculation. Where this occurs in the results chapters the upper confidence interval is described as not calculable and presented as n/a.

Mean survival time is estimated by the area under the survival curve and is based upon the entire range of data. It is possible to calculate the mean even if the survivor function remains greater than 0.5. The standard error of the mean was calculated using calculated using Greenwood's formula (Equation 3)[348] is also presented.

$$var\left(\widehat{S}(t)\right) = \left[\widehat{S}(t)\right]^2 \sum_{j:\tau_j<t} \frac{d_j}{(r_j-d_j)r_j}$$

Equation 3: Greenwoods formula for standard error of the mean (Kaplan-Meier analysis).

Where $\hat{S}$ = an estimate of the survival function
t = duration of study
τ_j = duration of study at point j when an event is sustained
d_j = number of events up to point j,
r_j = number of individuals at risk just prior to t_j

Average yearly freedom from event time was generated (Equation 4) to allow comparison to previously published literature and was done acknowledging this average does not always accurately reflect the actual freedom from event which was observed in the study.

$$\frac{\left(\frac{\#\ of\ individuals\ with\ an\ event\ in\ a\ group}{total\ number\ of\ participants\ in\ that\ group}\right)}{mean\ Kaplan-Meier\ freedom\ from\ event\ (years)}$$

Equation 4: Average annual event rate.

The continuous variables of cell count were statistically divided into tertiles to minimise the effect of non-normal distribution on Kaplan-Meir and Cox proportional hazards analysis. The categories of each cell type were derived with the following formula in S+:

> with(FinalAnalysis, quantile(celltype, 0:3/3, na.rm=T))

(where celltype = heading for each cell type continuous data column in the FinalAnalysis worksheet)

Kaplan-Meier log rank survival analysis was used to examine for statistical difference between disease severity at presentation groups for major adverse event Chapter 7. The Log rank test was used to assess whether the difference in disease severity groups were due to chance alone. The Log rank test is unable to adjust for other confounding factors but was performed prior to considering adjustment for disease severity in later Cox proportional hazards modelling of cell types and individual cardiovascular outcomes.

The association between cell types and major adverse events and death were tested in a stepwise manner testing each cell type with each outcome in turn. These results are presented separately in Chapter 8 Section 4 and 5. Each association was first assessed with Kaplan-Meier survival analysis. Comparison between two survival Kaplan-Meier curves should be based on formal non-parametric testing such as the log rank test, however the log rank test is unable to explore and adjust for the effects of confounding variables e.g.: age, risk factors for peripheral arterial occlusive disease, disease severity at presentation and medication usage and therefore was not used to assess the circulating cell types in this study. Adjustment for the confounding factors which are known to affect survival was considered preferable to simple analysis therefore Cox proportional hazards regression analysis was conducted instead of log rank testing.

4.4.2. Cox proportional hazards regression

Cox proportional hazards regression is the chosen method used to investigate the effect of several variables upon the time it takes for a specified event to take place. All Cox proportional hazard regression models were conducted using TIBCO Spotfire S+ 8.2 for Windows 2010

(TIBCO Software Inc. Boston, USA). A simplified version of the Cox proportional hazards model is presented in Equation 5.

$$\boldsymbol{\lambda(t,x) = \lambda_0(t)\,\exp(\beta x)}$$

Equation 5: Cox semi-parametric model.

Where λ(t,x) =hazard function that depends on time-point t,
$\lambda_0(t)$ =baseline hazard function that depends on time only,
exp (βx) =covariate related component

The Cox proportional hazards model also provides an estimate of the effect of each variable and allows an estimate of the hazard (or risk) of an event given a set value for prognostic variables which is not dependent on time (Equation 6). When there is a positive regression coefficient the hazard is higher and when there is a negative coefficient a better prognosis is implied with higher values for that variable. The hazard ratio (Equation 6) was obtained by comparing two groups at the same time (t), for example the Low TWCC Category and the Mid TWCC Category, or the Low TWCC Category and the High TWCC Category.

$$\boldsymbol{HR = \frac{\lambda(t,x_1)}{\lambda(t,x_2)} = \frac{\lambda_0(t)\,\exp(\beta x_1)}{\lambda_0(t)\,\exp(\beta x_2)} = \frac{\exp(\beta x_1)}{\exp(\beta x_2)}}$$

Equation 6: Hazard ratio.

λ(t,x) =hazard function that depends on time-point t,
$\lambda_0(t)$ =baseline hazard function that depends on time only,
exp (β x) =covariate related component
x_1 and x_2= are the covariate explanatory variables

Initial Cox analysis was *a priori* (without adjustment for confounding factors), followed by adjustment for well-established traditional clinical risk factors of age, sex, smoking, hypertension, diabetes mellitus and history of ischaemic heart disease or stroke (labelled traditional risk factors). This was done to enable comparison to previously published literature. The Cox proportional hazard always compared to the low circulating cell count group. Further

adjustment was made to include severity of disease status at presentation and medications aspirin and statins (labelled comprehensive risk factors). The evidence to include disease severity as a confounding variable presented in Chapter 7. As there is precedent for medication use to have significant effect on cardiovascular outcomes in patients with peripheral arterial occlusive disease, the comprehensive risk factor adjustment included the medications of aspirin and statin use as well as disease severity, age, sex, smoking, hypertension, diabetes mellitus, and history of ischaemic heart disease or stroke. When categorical co-variables were significantly associated with the outcome of Cox proportional hazards modelling the model was stratified for these variables and is presented in the results chapters as strata (variables/s). Stratification (Equation 7) allows the baseline hazard function to differ between subsamples and automatically excludes the stratification variables from explanatory data sets.[349]

$$\boldsymbol{\lambda_s(t,x) = \lambda_{S0}(t)\,\exp(\beta x)}$$

Equation 7: Stratified Cox proportional hazards model.

where s = 1, 2.., S – number of stratum
$\lambda_s(t,x)$ =hazard function that depends on time-point t for stratum s,
$\lambda_{S0}(t)$ =baseline hazard function that depends on time only for strum s,
exp ($\beta\, x$) =covariate related component

The assumptions of the Cox model were tested to ensure that the model could be appropriately applied to the data. Non-informative censoring bias was minimised during data collection. Care was taken in the recording of clinical data to ensure that discharge from follow up was not related to the probability of an event occurring. This was done via several means: thorough review of the patient's medical records (not only those related to vascular surgery), searching for admission to other Queensland Health hospitals for admission related to vascular condition or major adverse event and searching for record of the patient's death. The Cox proportional hazard assumption that the hazard function remains proportional over time was tested

statistically using the Cox.zph function. This function tests proportionality plots of the Schoenfeld[350] residuals versus log(time) and gives a Cox.zph value of significance that tests the proportional nature of the predictors tested. If the Cox.zph is significant then the time dependent covariates are not proportional with the potential implication that the assumption of the Cox model that relative hazard remains proportional over time may be breached. The global zph had the same function but instead of being applied to each variable is applied to the model as a whole. This violation should be taken into account and if possible the appropriate modification of the model should be used to enable precise interpretation.[351]

This study does use multiple planned comparisons between individual cell types and each outcome respectively. These planned comparisons are complementary in nature and as such no correction for multiple comparisons was indicated or applied.[352,353]

4.4.3. Multi-model averaging

Further statistical analysis uses the Information-Theoretical approach[132] to compare multiple models for each outcome. Multi-model averaging is considered the most useful technique for exploring a range of variables that may be associated with a particular outcome[132] and was conducted using statistical package R version 3.2.0.[354] The multi-model averaging package used was MuMIn: Multi-Model Inference. R package version 1.13.4.[355]

Traditional null hypothesis testing using stepwise regression has been commonly used for biological modelling using threshold based removal and introduction algorithms to obtain the best fit model when no terms can be added or removed. Stepwise regression results in a final model that does not contain any weak interaction terms and contains variables that are either

strong predictors or involved with strong interaction.[356] Although stepwise regression has been used commonly, it has many limitations including multiple testing[357] and the trade-off between model complexity and accuracy of parameter estimates.[356] In stepwise regression, variables can appear as significant or non-significant depending on which other variables are present in the model.[304,305] This is especially true when many interacting terms are used, particularly if these terms have small effects.[301] Stepwise regression also is unable to compare models and does not have model likelihoods and estimates of precision.[306] Stepwise regression is also unable to account for uncertainty linked to the process of selecting the final model, providing less than ideal coverage and narrow confidence intervals.[306] The limitations of stepwise regression have raised questions over its applicability for complex modelling in other scientific disciplines including ecology and the behavioural sciences.[358-362] Stepwise regression is not an appropriate analysis for this dataset due to multiple inter-related variables with small effects potentially creating severe bias and over or under estimation of the effect of variables.[302] Stepwise regression in similar datasets regardless of protocol has the potential to miss true predictors and identify significance for unimportant predictors.[303]

The Information-Theoretical approach, is well described by Burnham and Anderson[132] who explain that this approach which arises from likelihood theory compares multiple competing models at once by asking "how certain are we that any given model is the best approximating model?"[363] The process of which quantifies model selection uncertainty to give the model, from within the model set, that loses the least information - and gives the best approximation of the truth.

The Information-Theoretical approach to multi-model averaging was selected for this study because it is a complex model with proven interactions between variables and there are small effects of some or all of the variables.[302] To the author's knowledge, this is the first time the Information-Theoretical approach has been applied to risk factor modelling for patients with peripheral arterial occlusive disease.

4.4.3.1. The process of multi-model averaging

Akaike information criterion (AIC, Equation 8[364]) is used to compare multiple competing models all at once by asking "how certain are we that the given model is the best approximating model for the *true* process underlying the outcome being investigated?"[363] In doing so model uncertainty is quantified and accounted for through the assessment of the Kullback-Leibler information.[365] Kullback-Leibler information is the absolute distance of models from the biological truth,[132] allowing multiple competing models to be compared and ranked according to their relative distance from the truth. Ranking competing models by AIC allows them to be sorted in terms of information loss in approximating the unknowable truth.[306]

$$AIC = -2\log\left(\mathcal{L}\left(\hat{\theta}\middle|data\right)\right) + 2K$$

Equation 8: Akaike information Criterion

Where AIC = Akaike information Criterion,
$\log(\mathcal{L}(\hat{\theta}|data)$ = maximised log-likelihood over the unknown parameters (θ)
(The coefficient -2 applied for historical reasons)[364]
K = number of parameters estimated in that model

AICc is used to approximate AIC (Equation 9[364]) when n/k is less than 40 (where n is the number of models and k is the number of fitted parameters in the most complex model).[366]

$$AIC_c = \mathrm{AIC} + \frac{2\mathrm{K}(\mathrm{K}+1)}{\mathrm{n}-\mathrm{K}-1}$$

Equation 9: Akaike information Criterion corrected for sample size.

Where AICc = corrected Akaike information Criterion,
AIC = Akaike information Criterion,
K = number of parameters estimated in that model
n = the number of models

AICc is thus expanded for calculation with Equation 10[132]:

$$AIC_c = -2log\left(\mathcal{L}\{\hat{\theta}|data\}\right) + 2K + \frac{2K(K+1)}{(n-K-1)}$$

Equation 10: Akaike information criterion corrected for sample size calculation.

AIC_c = corrected Akaike information Criterion,
$\log(\mathcal{L}\{\hat{\theta}|data\})$ = maximised log-likelihood over the unknown parameters (θ)
(The coefficient -2 applied for historical reasons)[364]
K= total number of estimate parameters of the model,
n = the number of models,

The individual AIC values (or in this case AIC_c values) are not interpretable as they contain arbitrary constants and greatly affected by sample sizes.[132] AIC_c takes into account how well a model fits the data but models with a larger number of fitted parameters (k) will have larger AIC_c values. Each AIC_c is useless on its own, deriving it's meaning from comparison with the AIC_c values of other models, with the model having the lowest AIC representing the "best approximating model". The AIC_c values are rescaled such that the model with the minimum AIC_c has a value of 0 and are represented as Δ_i. [367]

$$\Delta_i = AIC_i - AIC_{min}$$

Equation 11: Δ_i or relative AIC

Δ_i = Relative AIC_c for model *i*
AIC_i = corrected Akaike information Criterion for model *i*
AIC_{min} = minimum corrected Akaike information Criterion,

The Δ_i values are easy to interpret and allow strength of evidence comparison and ranking of candidate models. The larger Δ_i is for any given model *i*, the less plausible the fitted model can be the best approximating model within the candidate set.[360]

The likelihood of an individual model g_i is expressed in Equation 12 and in the results chapters the log of this value is displayed log($\mathcal{L}$).

$$\mathcal{L}(g_i|data) = exp(-\Delta_i/2)$$

Equation 12: Likelihood of model g_i

Where g_i = model *I* *(for i = 1,2… R,)*
Δ_i = Relative AIC_c for model *i* (Equation 11)
exp (-½Δ_i) = transformation of Δ_i to provide the likelihood of the model, given the data

The Akaike weight w_i is to be interpreted as the probability of the model *i* being the best approximating the data set. Akaike weights measure the weight of evidence that model *i* is the actual Kullback-Leibler best model in the set and it is convention these values sum to 1 so w_i can be used as a probability.[367] The scaled model weights can then be used to rank the models, quantifying the evidence favouring one model over another.

$$w_i = \frac{exp(-\Delta_i/2)}{\sum_{r=1}^{R} exp(-\Delta_r/2)}$$

Equation 13: Akaike weight

Where w_i = Akaike weight for model *i* *(for i = 1,2… R,)*
R = number of models
Δ_i = Relative AIC_c for model *i* (Equation 11)
exp (-½Δ_i) = transformation of Δ_i to provide the likelihood of the model, given the data

Multi-model inference is using a set of models rather than any single model on which to base inference of parameter estimates which has both practical and philosophical advantages.[132,360]

Multi-model inference is done by calculating the cumulative evidence for the models containing a particular parameter combined with an estimate of the explained variation.[132] A model averaged estimator obtained from multi-model inference gives that estimator better precision and reduced bias over the estimator of that parameter from the selected best model alone.[360]

The models in this study that were not significantly different to the "best" model were selected using the Δ_i <2 function by convention.[132] Subsequently multi-model prediction is performed by averaging all the models with Δ_i <2, which estimates the relative influence of the potential predictors (labelled standardised coefficients) thus estimates the multi-model effects of determinants with respective standard error. The goal of this process is to generate an approximating model that accurately predicts the information in the entire data set after separation from the noise.[360] The standardised coefficients were then divided by their own standard error to generate a z score and subsequent p values. The interpretation of these p values is somewhat different to null hypothesis testing science such as Cox proportional hazards and stepwise regression. The use of these p value is are not traditionally part of the information theoretic approach[368]; they are examined with reference to each data set in respective results chapters.

The common terms used when describing and discussing the information-theoretic approach described above are summarised for ease of reference in Table 4.3.

Table 4.3: Common terms and definitions in multi-model analysis

Term	Description	Equation	Summary explanation
AIC	Akaike Information Criterion	Equation 8: $-2\log\left(\mathcal{L}(\hat{\theta}\|data)\right) + 2\mathrm{K}$	Quantifies information lost in approximating the biological truth (adjusted for fit and model complexity). Used to compare and rank different models.
AIC_c	Akaike Information Criterion corrected for sample size	Equation 9: $\mathrm{AIC} + \frac{2\mathrm{K}(\mathrm{K}+1)}{\mathrm{n}-\mathrm{K}-1}$	Used to approximate AIC for small sample size. Used in place of AIC for this thesis.
		Equation 10: $-2log\left(\mathcal{L}\{\hat{\theta}\|data\}\right) + 2K + \frac{2K(K+1)}{(n-K-1)}$	
$\boldsymbol{\Delta_i}$	Δ_i or relative AIC	Equation 11: $AIC_i - AIC_{min}$	The difference between model score and the "best" model. Facilitates comparison and ranking of models. Best model Δ_i =0
$\boldsymbol{\mathcal{L}}$	Likelihood of model g_i	Equation 12: $exp(-\Delta_i/2)$	Transformation of Δ_i to give the likelihood of a model.
log($\boldsymbol{\mathcal{L}}$)	Log likelihood of model g_i	$\log(exp(-\Delta_i/2))$	Measures lack of fit of model to the observed data.
w_i	Akaike weight	Equation 13: $\frac{exp(-\Delta_i/2)}{\sum_{r=1}^{R} exp(-\Delta_r/2)}$	Probability that model is the best in the data set. Total of all w_i =1 Quantifies evidence comparing models.
df	Degrees of freedom of model	Also referred to in the above equations as K	The number of independent values that can be assigned within a given model.
z score	Standardised coefficients divided by standard error	$\frac{Standardised\ coefficient}{Standard\ error\ of\ coefficient}$	Used to describe strength and robustness of coefficients.
p value	Quantifies strength of coefficient effect	R version 3.2.0.[354] used	Quantifies strength of coefficient effect.

4.4.4. Power Analysis

Preliminary analysis of previously collected data indicated a major adverse event rate of approximately 30% which in this group of 632 selected patients would be conservatively expected to yield 180 major adverse events. The sample size estimate was based on the work of Peduzzi (Equation 14).[369,370]

$$N = 10\ k / p$$

Equation 14: Required sample size for Cox proportional hazards.

where N = minimum number of cases to include,
p = the proportions of negative events (major adverse event) in the population,
k = the number of predictor variables

This study will used age, sex, smoking, hypertension, history of ischaemic heart disease, history of stroke, diabetes mellitus, disease severity at presentation, aspirin and statin as predictor variables (i.e. k=10) and the proportion of major adverse event in the population (p) is 0.30 (30%) from previous experience with the study population. [371] The minimum number of cases predicted to be required was therefore 333 (Equation 15). The study was designed and powered for the primary outcome of major adverse event.

$$N = 10 \times 10 / 0.30 = 333$$

Equation 15: Required sample size for this study for outcome of major adverse event.

Where N = minimum number of cases required

Consideration was then given to ensure adequate power for the secondary endpoint of death where the incidence of death in the population with intermittent claudication is expected to be in the range of 4-6% per year, higher in those with more severe disease.[24,25,29,49,72,222] From previous experience with the study population (p) was estimated to be ~0.25 (25%). [371]

$$N = 10 \times 10 / 0.25 = 400$$

Equation 16: Calculation of required sample size for secondary outcome of death alone.
Where N = minimum number of cases required

It was acknowledged from the outset that the endpoints of heart attack, stroke and major amputation were unlikely to contain sufficient events to provide sufficient power for independent testing.

In multi-model analysis it has been accepted as convention that a sample size 'rule of thumb' of 10:1 subjects to predictors be applied.[132,372,373] This study was adequately powered to examine the 15 predictors included in the multi-model analysis consisting of the 10 variables listed above combined with Neutrophil Category, Lymphocyte Category, Monocyte Category, Haemoglobin Category and calculated Neutrophil/Lymphocyte Ratio (NLR) Category.

Sufficient participants were recruited based on sample size estimates for the outcomes of major adverse event and death and allowed for rigorous application of inclusion and exclusion criteria to the 632 potential participants (described in detail in Chapter 5).

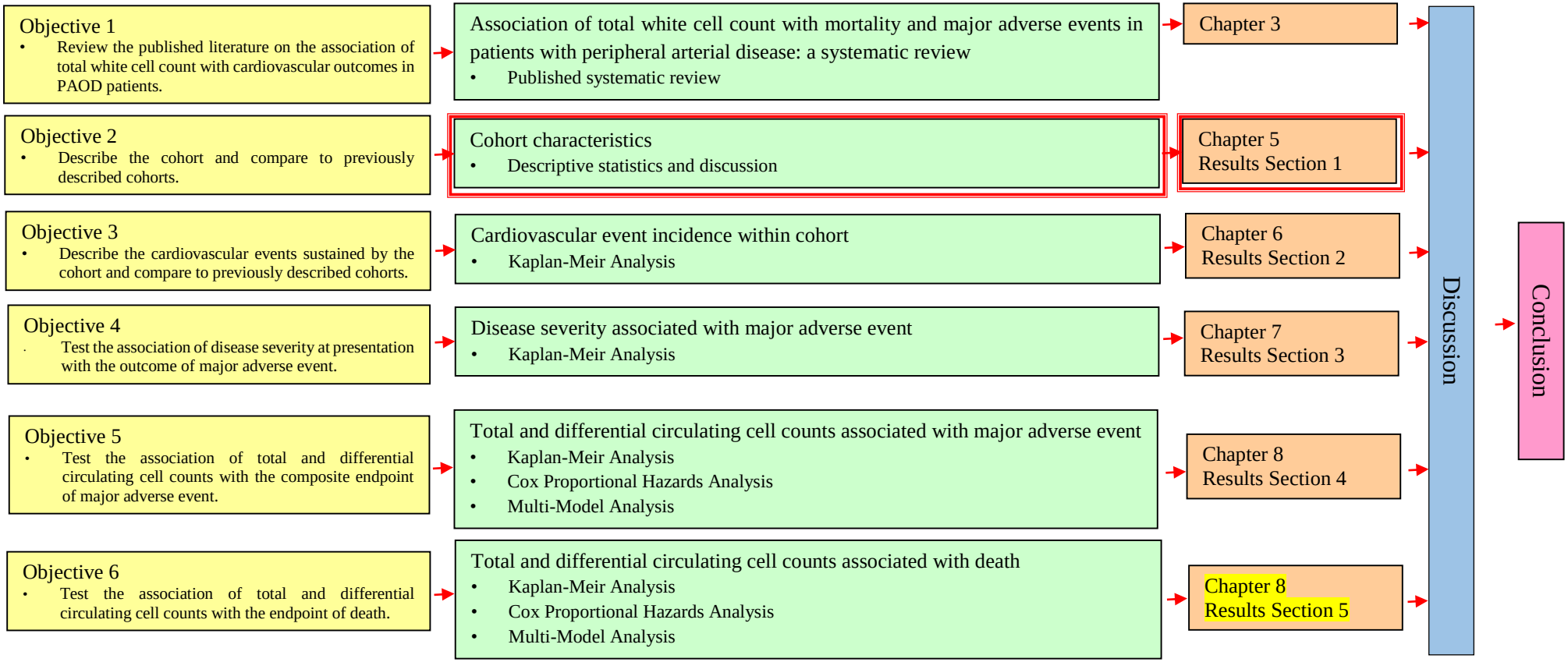

Figure 5.1: Schematic overview of thesis with red box highlighting current position within document – cohort characteristics

5. Results - section 1

Cohort characteristics

The characteristics of patients with peripheral arterial occlusive disease vary with the population assessed. Prior to investigating the outcomes for this patient cohort with symptomatic peripheral arterial occlusive disease it is important to examine the characteristics of the cohort and compare to other populations of patients with peripheral arterial occlusive disease. Chapter 5 will set the scene for the following results chapters by describing and examining the characteristics of this cohort of patients with peripheral arterial occlusive disease at recruitment. This cohort will then be compared to previously investigated cohorts with symptomatic peripheral arterial occlusive disease.

The detailed methodology for this results section is described in Chapter 4. The selection of the cohort is presented with exclusion and inclusion criteria results. The characteristics of the cohort are presented in tabular form and compared to previously described populations with this disease.

5.1. Inclusion and exclusion results

A total of 632 patients were considered for inclusion into the study with 398 patients eligible for inclusion in the final analysis. The most common reason for exclusion was the absence of symptomatic peripheral arterial occlusive disease (n=158), followed by no pre-operative or study baseline bloods (n=27) and less than one month follow up (n=18) with the complete list of exclusions included in Figure 5.2

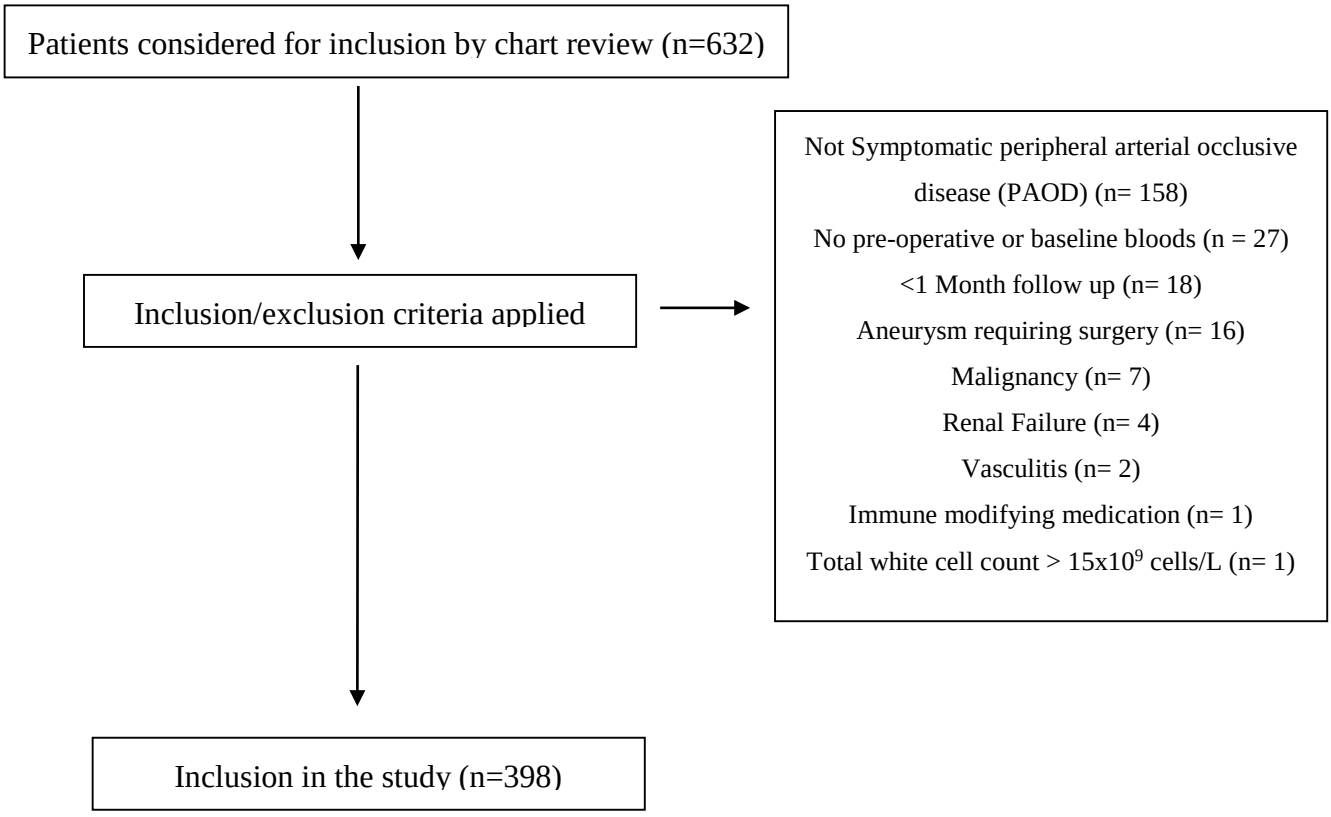

Figure 5.2: Flow diagram of patients included in this study

5.2. Characteristics of the cohort

The mean age of the cohort was 66.34 (±9.81) years ranging from 38 to 95.9 years at recruitment (Table 5.1). The indication for inclusion to the study at presentation was intermittent claudication in 291 patients (73.1%), tissue loss in 59 patients (14.8%), rest pain in 39 patients (9.8%) and acute lower limb ischaemia was less represented with only 13 patients (3.3%). The risk factors and medication use of the total cohort and each disease severity at presentation group are presented in Table 5.1. The Tissue Loss group had the highest proportion of never smokers (37.3%) compared to the Rest Pain group (11.4%), the intermittent claudication group (10.7%) and the Acute Lower Limb Ischaemia group (7.7%). Current smokers were more common in the Rest Pain (54.3%) and Acute Lower Limb Ischaemia (53.8%) groups than the Intermittent Claudication group (47.1%) and the Tissue Loss group (42.4%). The largest proportion of Ex-smokers was in the Intermittent Claudication group (38.2%) with the smallest proportion of ex-smokers in the Tissue Loss group (20.3%).

While the presence of diabetes mellitus in the total cohort was 37.4%, there was a larger representation of diabetics in the Rest Pain group with 79.7% of the Rest Pain group diagnosed with diabetes or taking medication for glycaemic control. The Acute Lower Limb Ischaemia group had the lowest proportion of diabetes with 23.1% of this population diagnosed or under treatment for this condition. The Intermediate Claudication, Rest Pain and Tissue Loss groups had a similar percentage of participants diagnosed with ischaemic heart disease or previous heart attack to that of the overall cohort ~45%. The Acute Lower Limb Ischaemia group was lower with only 30% of this group known to have ischaemic heart disease or previous heart attack.

Table 5.1: Characteristics of the cohort

Characteristic	Total Cohort (n=398)	Disease Severity at Presentation			
		Intermittent Claudication (n=291)	Tissue Loss (n=59)	Rest Pain (n=35)	Acute Lower Limb Ischaemia (n=13)
Age (years)	66.3 (±9.8)	66.1 (±9.5)	69.1 (±10.3)	64.2 (±10.8)	65.8 (±11.7)
Male gender	278 (69.8%)	214 (73.5%)	34 (57.6%)	19 (54.3%)	11 (84.6%)
Never Smoker	58 (14.6%)	31 (10.7%)	22 (37.3%)	4 (11.4%)	1 (7.7%)
Current Smoking	188 (47.2%)	137 (47.1%)	25 (42.4%)	19 (54.3%)	7 (53.8%)
Ex-smoking	152 (38.2%)	123 (42.3%)	12 (20.3%)	12 (34.3%)	5 (38.5%)
Hypertension	298 (74.9%)	219 (75.3%)	47 (79.7%)	25 (71.4%)	7 (53.8%)
Diabetes	149 (37.4%)	91 (31.3%)	43 (79.7%)	12 (34.3%)	3 (23.1%)
Previous MI/IHD	177 (44.5%)	131 (45%)	26 (44.1%)	16 (45.7%)	4 (30.8%)
Stroke	36 (9.0%)	28 (9.6%)	5 (8.5%)	2 (5.7%)	1 (7.7%)
TIA	35 (8.8%)	31 (10.7%)	1 (1.7%)	3 (8.6%)	0 (0%)
Prev Endovascular	55 (13.8%)	40 (13.7%)	8 (13.6%)	5 (14.3%)	2 (15.4%)
Prev Open	49 (12.3%)	30 (10.3%)	11 (18.6%)	4 (11.4%)	4 (30.8%)
Aspirin	276 (69.3%)	207 (71.1%)	37 (62.7%)	23 (65.7%)	9 (69.2%)
Other Antiplatelet	53 (13.3%)	45 (15.5%)	5 (8.5%)	3 (8.6%)	13 (100%)
Warfarin	31 (7.8%)	21 (7.2%)	6 (10.2%)	3 (8.6%)	1 (7.7%)
CCB	111 (27.9%)	81 (27.8%)	17 (28.8%)	11 (31.4%)	2 (15.4%)
Beta-blockers	114 (28.6%)	89 (30.6%)	14 (23.7%)	7 (20.0%)	4 (30.8%)
ACE-inhibitors	167 (42.0%)	122 (41.9%)	29 (49.2%)	13 (37.4%)	3 (23.1%)
Ang II blockers	90 (22.6%)	64 (22.0%)	19 (32.2%)	5 (14.3%)	2 (15.4%)
Frusemide	50 (12.6%)	28 (9.6%)	17 (28.8%)	5 (14.3%)	0 (0%)
Other Diuretic	52 (13.1%)	39 (13.4%)	10 (16.9%)	2 (5.7%)	1 (7.7%)
Statins	259 (65.1%)	189 (64.9%)	37 (62.7%)	25 (71.4%)	8 (61.5%)
Fibrate	14 (3.5%)	10 (3.4%)	1 (1.7%)	3 (8.6%)	0 (0%)
NSAIDs	63 (15.8%)	47 (16.2%)	10 (16.9%)	5 (14.3%)	1 (7.7%)
Cox2	38 (9.5%)	29 (10.0%)	6 (10.2%)	2 (5.7%)	1 (7.7%)
Metformin	103 (25.9%)	65 (22.3%)	29 (49.2%)	9 (25.7%)	1 (7.7%)

ACE inhibitors = angiotensin converting enzyme inhibitor (antihypertensive medication)
Ang II blockers = angiotensin II receptor antagonist (antihypertensive medication)
CCB = calcium channel blocker (antihypertensive medication)
Cox2 = Cox 2 receptor selective non-steroidal anti-inflammatory drug
MI/IHD = myocardial infarction (heart attack) or ischaemic heart disease
NSAIDs = non-steroidal anti-inflammatory drugs
Prev Endovascular = previous endovascular intervention
Prev Open = previous open revascularisation
TIA = transient ischaemic attack

The previous occurrence of stroke was between five and ten percent in all groups, with nine percent of the overall cohort having a previous stroke. The Rest Pain group had the lowest proportion of previous strokes with only 5.7%. The prevalence of previous transient ischaemic attack in the cohort was 8.8%, with a higher percentage in the Intermittent Claudication group (10.7%) and the Rest Pain group (8.6%) than the Tissue Loss group (1.7%) with no previous transient ischaemic attacks in the Acute Lower Limb Ischaemia group.

Previous endovascular intervention was relatively consistent across all disease severity at presentation groups with rates from 13.6 – 15.4%. Previous open revascularisation was more common in the Acute Lower Limb Ischaemia group (30.8%) compared to 10.3% of the Intermittent Claudication group and 11.4% of the Rest Pain group.

Aspirin was used in 69.3% of the cohort at recruitment, and was similar across disease severity at presentation groups. Aspirin was most commonly used in the Intermittent Claudication group (71.1%) and Acute Lower Limb Ischaemia group (69.2%) with the lowest frequency of aspirin use in the Tissue Loss group (62.7%) and in the Rest Pain group (65.7%). Other antiplatelet drugs were used in all of the Acute Lower Limb Ischaemia patients, meaning that 69.2% of this population were on dual antiplatelet therapy. In the Intermittent Claudication group other antiplatelet use was 13.3% with similar rates in the Tissue Loss (8.5%) and Rest Pain (8.6%) groups.

5.3. Discussion

The mean age of this cohort is within ten years of the mean age of all of the cohorts that reported age in Chapter 3.[223] This population is however younger than six[49,74,122,222,320,322] of those

nine[24,49,72-74,122,222,320,322] populations. The Tissue Loss group in this study had a mean age three years older than the Intermittent Claudication group and almost five years older than the Rest Pain group (Table 5.1).

Variation is seen in the gender mix of disease severity at presentation groups with 69.8% of the total cohort male but 51% of the Rest pain group and 43% of the tissue loss group female (Table 5.1) consistent with previous description of higher representation of females in the critical limb ischaemia population.[9] There is a range of variability in the gender of previously examined cohorts varying between 50-93% male gender with six studies[72,74,122,320-322] reporting between 60-80% male gender with this study population in the middle of that range.

A larger percentage of the studied cohort are current smokers (47.2%) than five[49,72,73,122] of the comparative studies. The current and ex-smoking groups combined make up 85.4% of the total cohort ("smokers") which is greater than all the reported incidence of smoking in Chapter 3[223] in the studies that included both current and ex-smokers in this group.[12,74,322]

The risk factor of hypertension was present in 74.9% of the cohort which is comparable to five[72,74,122,320,322]of the eight studies that reported this information. Arain et al.[222] reported a lower incidence of hypertension (66%) while Dormandy and Murray[24] reported a lower incidence again (57%) and Violi et al.[73] a much lower incidence with only 35% of the patients in that cohort having hypertension. It is possible that the definition of hypertension plays a part in the observed variation between populations with the patients in this study recorded as having the risk factor of hypertension if they were on anti-hypertensive medication even if their blood pressure was normal.

Diabetes was present in 37.4% of the examined cohort which is a similar result to that of two studies,[72,322] but less than that of one study[122] with 51-57% of their population diabetic. This cohort had more diabetics than two studies with ~25%[222,320] and two studies with less (14%[24], 19%[73]).

Previous heart attack or ischaemic heart disease was present in 44.5% of the total cohort which is comparable to two studies[74,222] and consistent with the consensus of population studies of ~40%[28]. This cohort had significantly more heart disease than two other studies with 1.9-2.5%[320] and 10%[24] having ischaemic heart disease. It is unclear from the published methodology why the population of Amaranto et al.[320] would have a rate of ischaemic heart disease so much lower than that generally expected of a population with peripheral arterial occlusive disease although diagnosis can greatly depend on the sensitivity of methods used.[28]

Tissue Loss was present on admission to the study in 14.8% of the total cohort, almost double the 7.8% concentration in the cohort of Barani et al.[122]. Rest pain was present in 9.8%. Three studies[24,73,74] only included patients with intermittent claudication while the other populations did not report the severity of peripheral arterial occlusive disease of included cases. In this study there were 94 patients in the combined groups of Rest Pain and Tissue Loss, which would give 24.6% of this cohort having critical limb ischaemia at presentation. This is similar to the 22.3% of the Haumer et al.[72] cohort of exactly the same size. Of the critical limb ischaemia patients in this study 62.8% had tissue loss, less than the 75% in the Pedrinelli et al.[322] cohort which only included patients with critical limb ischaemia.

Previous interventions and medication use were not reported consistently enough in comparable populations to enable valuable comparison. Thorough inclusion of cohort characteristics in future research will allow for more accurate comparison and compilation of results. It is recommended that this include not only incidence of traditional risk factors but disease severity and medication use.

5.4. Conclusion

Definitions of risk factors for the included populations with peripheral arterial occlusive are not standardised and only sometimes published[223] making comparison between populations and compilation of like populations for meta-analysis problematic. This study population of peripheral arterial occlusive disease patients does have a greater number of smokers compared with the compared previous populations and does have a comparatively high record of comorbidities overall.

This cohort of patients with peripheral arterial occlusive disease is suitable for comparison to the other populations with peripheral arterial occlusive disease although caution needs to be taken in applying the outcome analysis from this population directly to other populations with peripheral arterial occlusive disease as there are inconsistencies in risk factors between populations. How much of this variation is due to methodological differences is difficult to ascertain as there are not currently standardised methods for assessing and recording risk factors in populations with peripheral arterial occlusive disease.

Comparison between populations of patients with peripheral arterial occlusive disease and collation of population data for meta-analysis would be significantly facilitated if standardised definitions of comorbidities were developed and employed for future research.

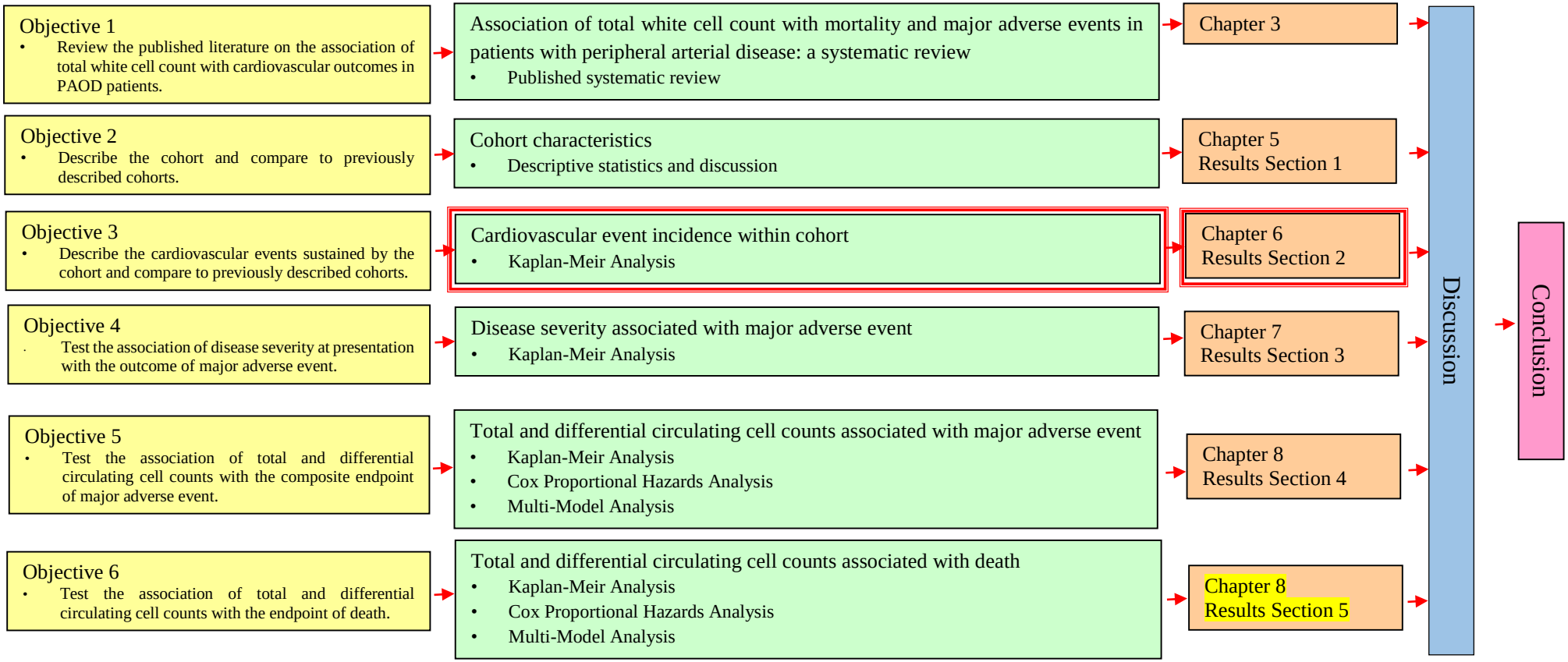

Figure 6.1: Schematic overview of thesis with red box highlighting current position within document – Cardiovascular event incidence within cohort

6. Results - section 2

Cardiovascular event incident in cohort of patients with peripheral arterial occlusive disease

Cardiovascular outcomes of patients with peripheral arterial occlusive disease vary depending on the population assessed. With variation in the comparison of reported cardiovascular risk factors shown in Chapter 5 it is important to establish the comparability of the cardiovascular event rate of the studied cohort to other populations with peripheral arterial occlusive disease. The principle aim of this chapter was to define and describe the incidence of major adverse event and the individual cardiovascular outcomes of death, heart attack, stroke, major amputation and peripheral revascularisation over the course of the study and then compare to previously published populations with peripheral arterial occlusive disease described in the systematic review in Chapter 3.[223]

The detailed methodology for the results presented in this section is available in Chapter 4. The time-point of 3500 days is shown consistently throughout the results chapters as no patients remained in the study alive at 4000 days (10.96 years). As outlined in Chapter 4 when the Kaplan-Meir survival function does not get below 0.5 the median is presented as n/a and when the confidence interval for the median or mean is unable to be calculated it is presented as n/a.

6.1. Cohort major adverse event (death, heart attack or stroke) Kaplan-Meier analysis

Kaplan-Meier survival plot of the freedom from major adverse event in the cohort is shown in Figure 6.2 with the dotted lines representing the 95% confidence intervals. The horizontal axis is displayed in days with 1000 days (2.74 years), 2000 days (5.48 years), 3000 days (8.22 years) and 3500 days (9.59 years). The shape of the Kaplan-Meier freedom from major adverse event plot graphically displays the steady rate of major adverse event observed over the duration of the study.

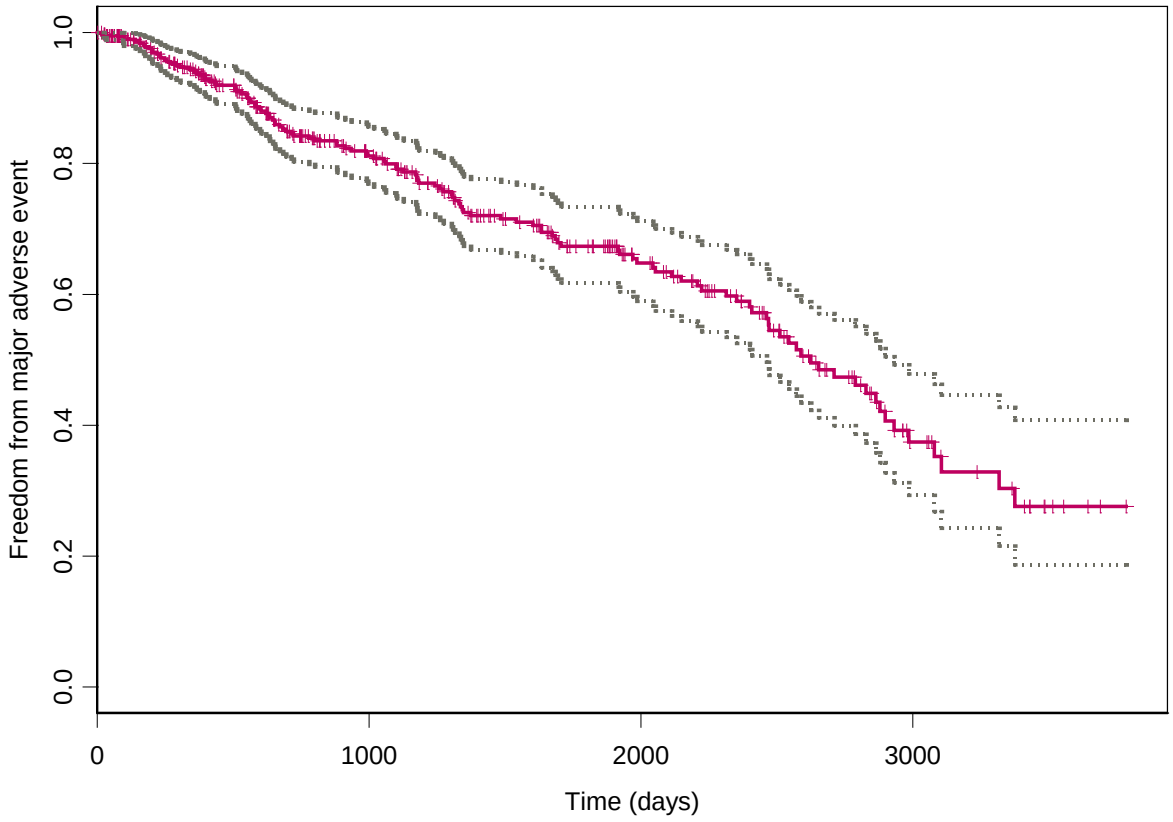

Figure 6.2: Kaplan-Meier non-parametric survival plot - freedom from major adverse event (death, heart attack or stroke) of cohort with 95% confidence intervals

The average yearly rate of major adverse events was calculated to allow comparison with other populations over variable follow up times and is 4.9% major adverse event per year, although the true rate is more uneven (Figure 6.2).

A total of 124 out of the 398 patients in the cohort either died or sustained a heart attack or stroke, giving an incidence of major adverse event of 35.16% over the course of the study. There were 269 patients discharged from follow up without major adverse event and a total of 503 237 patient follow up days (Table 6.1).

Median time to major adverse event was 2625 days (7.2 years) and is the preferred measure of central tendency for Kaplan-Meier analysis. The mean time to major adverse event of 2405 days (6.6 years) was calculated using the area under the curve function.

The progress of patients over time (Table 6.1) is shown with number of patients remaining in the study, the number of events and survivor function with 95% confidence intervals at each time point of 1000, 2000, 3000 and 3500 days.

Table 6.1: Kaplan-Meier freedom from major adverse event (death, heart attack or stroke) rate of cohort with 95% confidence intervals displayed by days follow up.

Major Adverse Event		0 days	1000 days	2000 days	3000 days	3500 days
Total Cohort	At Risk	398	205	98	20	5
	Events in previous interval	0	59	34	27	4
	Freedom from major adverse event (95% CI)	1	0.81 (0.77-0.86)	0.65 (0.59-0.72)	0.37 (0.29-0.48)	0.28 (0.19-0.41)

CI = Confidence Interval

In the first 1000 days 59 patients sustained a major adverse event with 134 patients discharged from follow up in this period leaving 205 patients at risk on day 1000 (Table 6.1). Of all the major adverse events that occurred during this study 47.6% of them occurred within the first 1000 days. At 2000 days, 98 patients remained at risk with a further 34 having sustained a major adverse event and a further 73 patients discharged from the study. By 3000 days only 20 patients remain at risk with 27 patients having sustained either death, heart attack or stroke in the previous 1000 days, and a total of 258 patients discharged from the study by this time. Between 3000 and 3500 days a further four major adverse events were sustained with only five patients remaining in the study at risk by day 3500, all of whom were discharged without sustaining a major adverse event prior to 4000 days.

The endpoint of major adverse event for each patient is the time from study entry to the first event of heart attack, stroke or death. The patients in the study may have had multiple cardiovascular events prior to their death and the endpoints of death, heart attack and stroke are presented individually in addition to the endpoints of major amputation and peripheral revascularisation to clarify these relationships and describe events sustained by the study population.

6.2. Cohort death Kaplan-Meier analysis

Kaplan-Meier survival plot of the freedom from death in the cohort is shown in Figure 6.3 with the 95% confidence intervals. During the 530 246 patient follow up days 100 patients of the total 398 patients in the cohort died with the remainder discharged from follow up over the course of the study. The terminal drop off in Figure 6.3 is due to the last remaining patient in the study dying at that time.

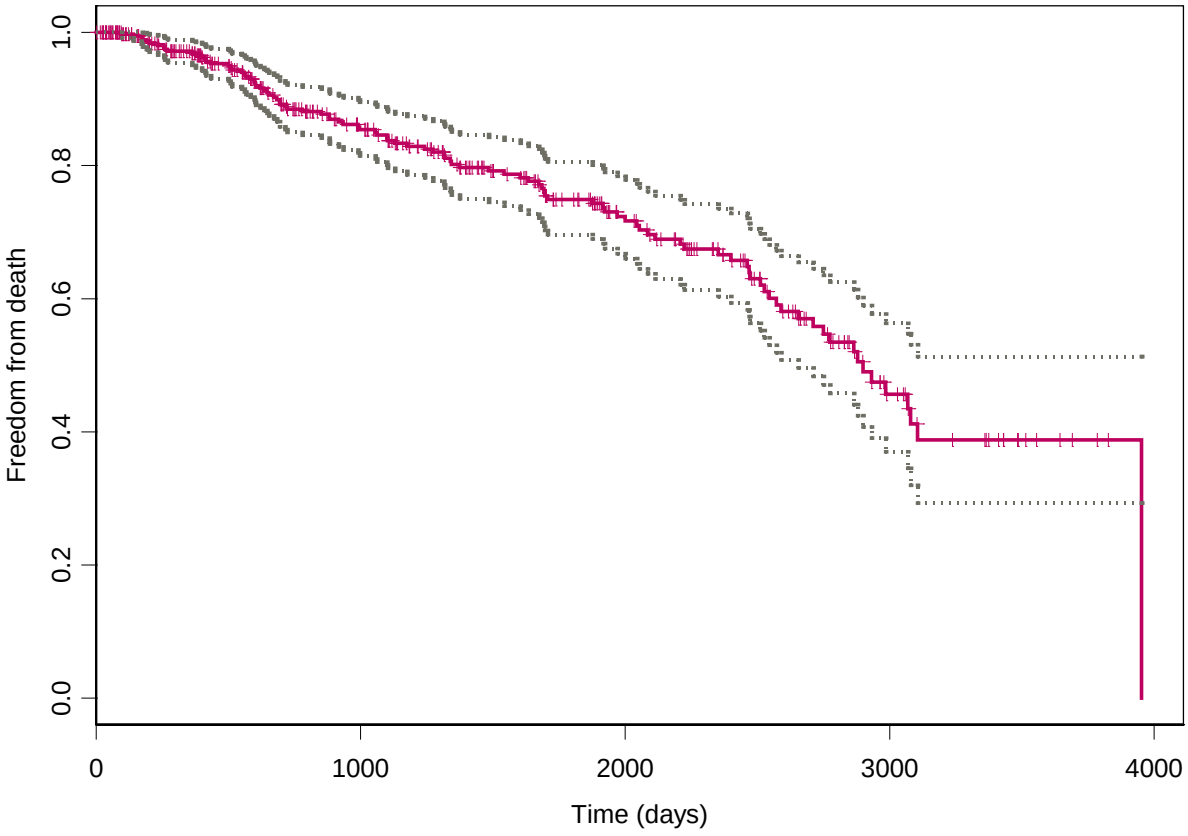

Figure 6.3: Kaplan-Meier non-parametric survival plot - freedom from death of cohort with 95% confidence intervals

The freedom from major adverse event curve (Figure 6.2) has a different end shape to the freedom from death curve (Figure 6.3). Although the same patient is included in both data sets and death was recorded at the same time point, only the first major adverse event is counted therefore that patient exits with a major adverse event (heart attack or stroke) prior to the recorded death. There is a noticeable change in the freedom from death function at around 3000 days with a flattening out of the curve (Figure 6.3) indicating a stabilising of the freedom from death function that had been increasing in gradient prior to 3000 days although with only 24 patients left at risk at 3000 days.

The overall incidence of death was 25.1% (total deaths 100) with median freedom from death 2899 days (7.9 years) and mean freedom from death more than 180 days less at 2715 days (7.4 years). The observed difference in median and mean freedom from death is due to the skewed nature of the freedom from death function. While it takes 2899 days for the median freedom from death to occur, the half of the population that survive to this time do not live as long after this point as they did prior (in this study) reducing the mean which is calculated using an area under the curve method.

The total mortality rate over the duration of the study is 25.1% with an average yearly mortality rate of 3.3% although this varies over the duration of the study (Table 6.2). The progress of patients over time is shown in Table 6.2 with the number of patients remaining in the study (at risk), the number of deaths and the Kaplan-Meier survivor function with 95% confidence intervals at each time point of 1000, 2000, 3000 and 3500 days.

Table 6.2: Kaplan-Meier freedom from death rate of cohort with 95% confidence intervals displayed by days follow up.

Death		0 days	1000 days	2000 days	3000 days	3500 days
Total Cohort	At Risk	398	215	108	24	7
	Events in previous interval	0	44	27	25	3
	Freedom from death (95% CI)	1	0.85 (0.82-0.90)	0.72 (0.66-0.78)	0.46 (0.37-0.56)	0.39 (0.29-0.51)

No. = number n/a = not applicable CI= Confidence Interval

398 patients began the study at day 0, by 1000 days only 215 patients were remaining in the study, with 44 patients dying prior to this and 139 patients being discharged from the study (Table 6.2). By 2000 days 108 patients remained in the study (at risk), with a further 27 patients deceased, and a further 80 patients discharged from follow up. At 3000 days only 24 patients remained in the study because between 2000 and 3000 days 25 patients died and 59 patients were discharged from follow up. At 3500 days there were seven patients remaining at risk (freedom from death 0.388) while three patients died in the prior 500 days. The last patient being followed up dies at 3952 days, therefore the freedom from death curve drops to 0 at this point (Figure 6.3). 3500 days is shown consistently throughout the results chapters as no patients remained in the study alive at 4000 days.

6.3. Cohort heart attack Kaplan-Meier analysis

Kaplan-Meier plot of the freedom from heart attack for the entire cohort is shown in Figure 6.4 with the dotted lines displaying the 95% confidence intervals. Of the 398 patients in the cohort only 34 patients sustained a heart attack over a total of 511 846 patient follow up days, making the freedom from heart attack survival curve (Figure 6.4) less steep than the freedom from major adverse event (Figure 6.2) and freedom from death (Figure 6.3) Kaplan-Meier plots presented previously.

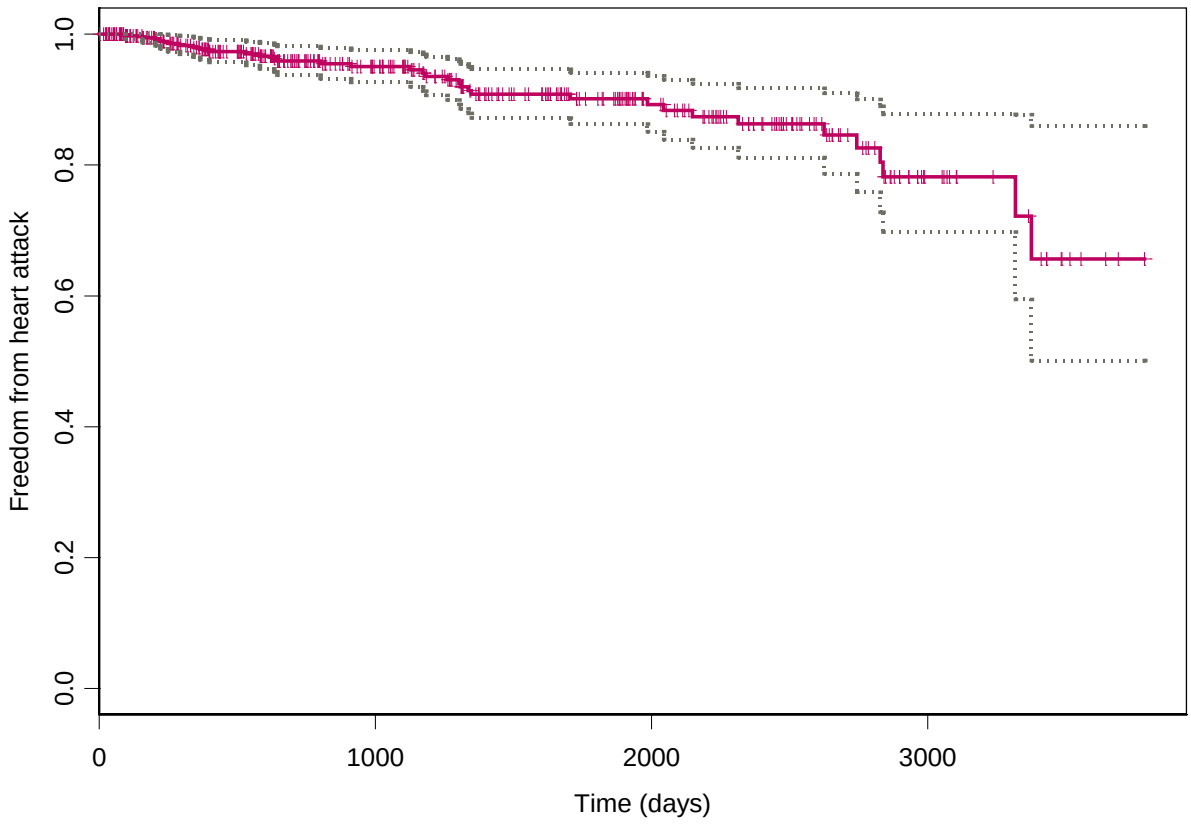

Figure 6.4: Kaplan-Meier non-parametric survival plot - freedom from heart attack of cohort with 95% confidence intervals.

The larger drops in the freedom from heart attack curve toward the end of the study (Figure 6.4) are because each heart attack is sustained in a smaller at risk population and has a greater effect on the freedom from heart attack function than heart attacks that were sustained early in the course of the study when there was a comparatively larger at risk population.

Heart attack was sustained by 34 patients with five patients remaining in the study at conclusion (therefore no terminal drop off in Figure 6.4). Discharge from follow up without heart attack occurred in 359 patients over the course of the study (Table 6.3).

Overall incidence of heart attack was 8.5%, occurring in 34 of the 398 patients, and the calculated mean freedom from heart attack by the area under curve method was 3299 days (± 78days) or 9.0 years. Median freedom from heart attack and 95% confidence intervals for the median were not applicable due to the relatively small number of heart attacks resulting in the freedom from heart attack curve not reaching 0.5.

The tabular presentation of progress of patients over time (Table 6.3) may be used to view the patients remaining at risk in the study, and when heart attacks occurred in relation to time posts 1000, 2000, 3000 and 3500 days.

Table 6.3: Kaplan-Meier freedom from heart attack rate of cohort with 95% confidence intervals displayed by days follow up.

Heart Attack		0 days	1000 days	2000 days	3000 days	3500 days
Total Cohort	At Risk	398	208	102	20	5
	Events in previous interval	0	15	10	7	2
	Freedom from heart attack (95% CI)	1	0.95 (0.93-0.98)	0.89 (0.85-0.94)	0.78 (0.70-0.88)	0.66 (0.50-0.86)

CI = Confidence Interval

Of the 34 heart attacks sustained by patients enrolled in the study, the majority of these events occurred in the first 2000 days with only 9 further events occurring after this time (Table 6.3). The two heart attacks that occur between 3000 and 3500 days result in much larger drops in the survival cure (Figure 6.4) as discussed above because there are markedly fewer (≤20) patients still at risk. This shows the importance of discharge or censorship of other patients that can also be ascertained from Table 6.3, prior to 1000 days 175 patients were discharged from follow up, with a further 96 discharged between 1000 and 2000 days, 75 between 2000 and 3000 days and 13 between 3000 and 3500 days.

6.4. Cohort stroke Kaplan-Meier analysis

Kaplan-Meier survival plot of the freedom from stroke curve for the entire cohort is shown in Figure 6.5 with the 95% confidence intervals displayed with the dotted lines. The flatter shape of the freedom from stoke plot is due to only 18 strokes being sustained over 519 695 patient follow up days.

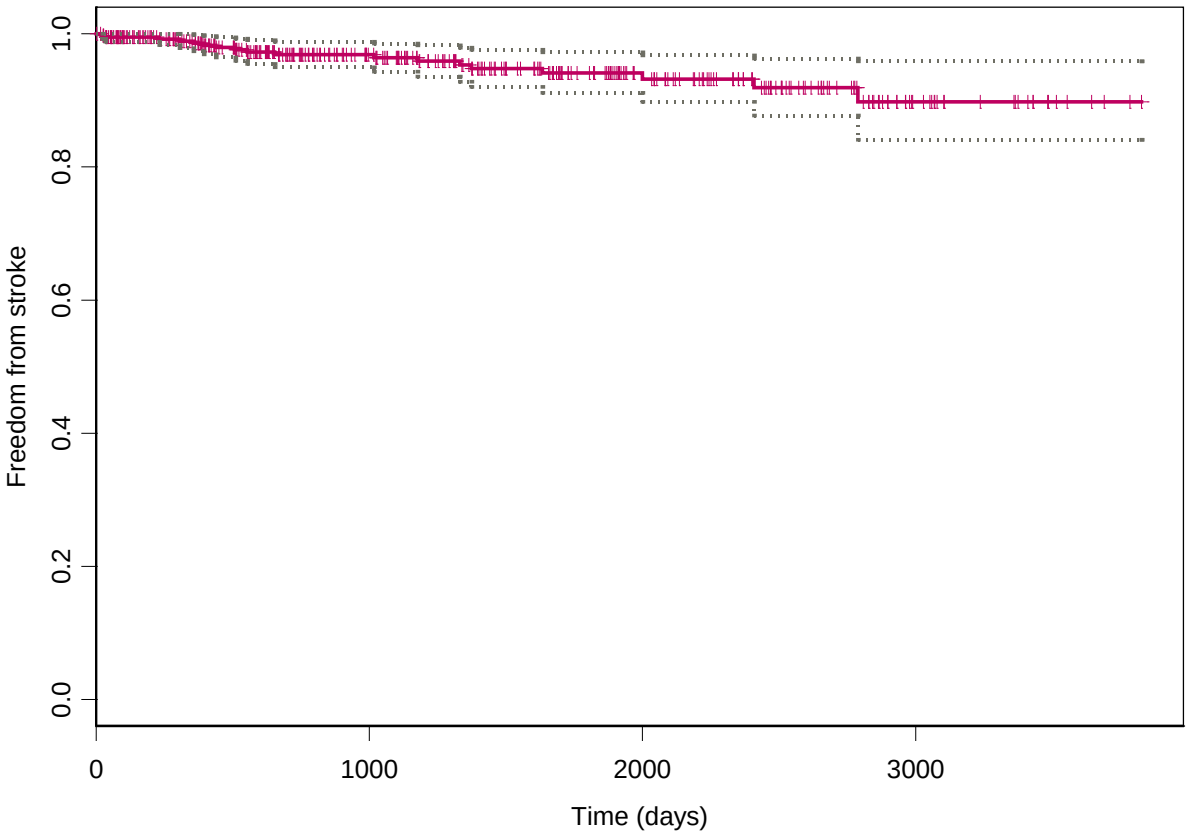

Figure 6.5: Kaplan-Meier non-parametric survival plot - freedom from stroke of cohort with 95% confidence intervals.

Mean time to stroke was calculated to be well over 3000 days or >9 years (with median not applicable), and should be interpreted with caution due to the small number of strokes (Table 6.4). Overall incidence of stroke was 4.5% of the recruited population.

The progress of patients over time (Table 6.4) is shown with number of patients remaining in the study, the number of strokes and survivor function with 95% confidence intervals at each time point of 1000, 2000, 3000 and 3500 days.

Table 6.4: Kaplan-Meier freedom from stroke rate of cohort with 95% confidence intervals displayed by days follow up.

Stroke		0 days	1000 days	2000 days	3000 days	3500 days
Total Cohort	At Risk	398	211	104	23	6
	Events in previous interval	0	10	6	2	0
	Freedom from stroke (95% CI)	1	0.97 (0.95-0.99)	0.93 (0.90-0.99)	0.90 (0.84-0.96)	0.90 (0.84-0.96)

CI = Confidence Interval

Ten of the 18 strokes (55.5%) occurred in the first 1000 days of the study (Table 6.4). There were six strokes that occurred between 1000 and 2000 days and two strokes between 2000 and 3000 days. The freedom from stroke rate does not change from 3000 to 3500 days as there were no recorded strokes during this time. Discharge from follow up without stroke occurred in 177 patients in the first 1000 days, 101 patients between 1000 and 2000 days, 79 patients between 2000 and 3000 days and 17 patients between 3000 and 3500 days.

6.5. Cohort major amputation Kaplan-Meier analysis

Kaplan-Meier survival plot of the incidence of major amputation in the cohort is shown in Figure 6.6 with the dotted lines displaying the 95% confidence intervals. The freedom from major amputation function shows gradual decline over the course of the study with one step down before flattening out prior to 3000 days (Figure 6.6).

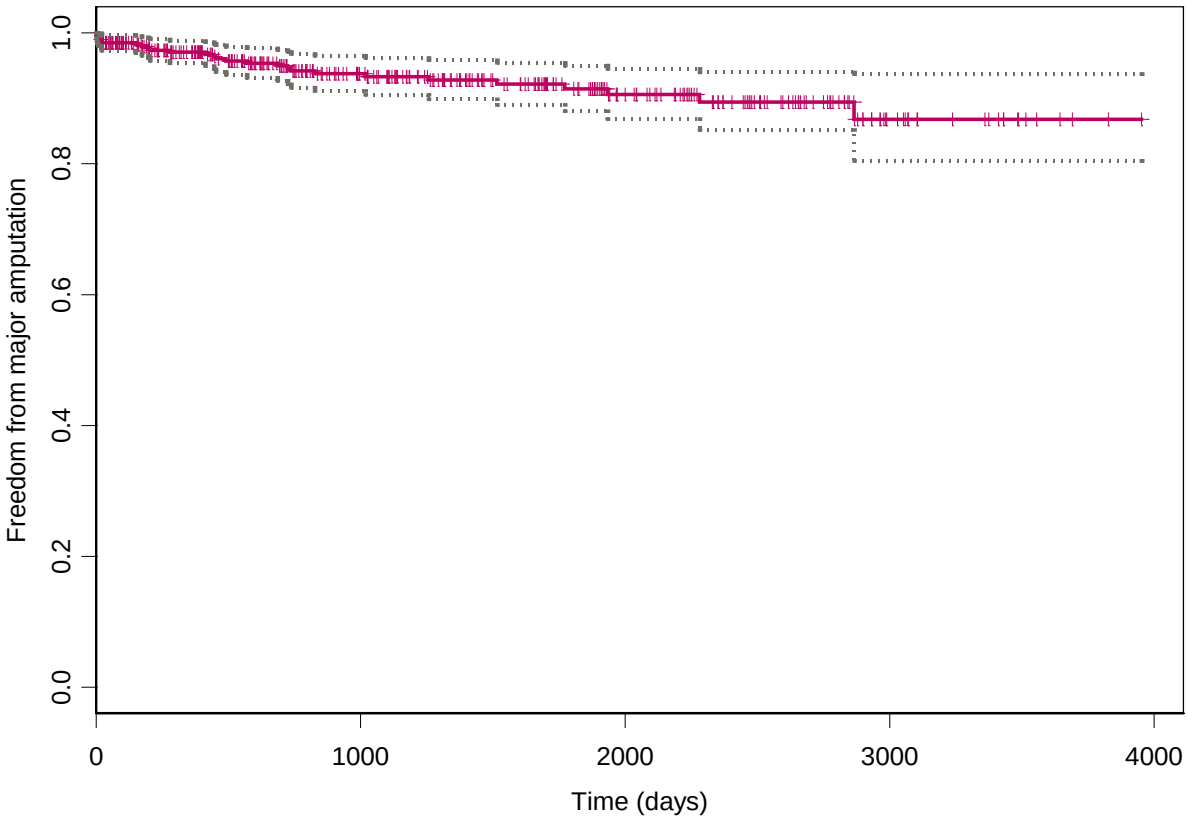

Figure 6.6: Kaplan-Meier non-parametric survival plot - freedom from major amputation of cohort with 95% confidence intervals

Of the 398 patients in the cohort 27 patients required major lower limb amputation and 365 were discharged from follow up with a total of 509 787 patient follow up days (Table 6.5).

The mean freedom from major amputation is calculated from the area under the curve method and is well over 3000 days or >9 years and again should be interpreted with caution due to the small number of major amputations and the median not being applicable. Overall incidence of major amputation was 6.8% of the study population.

The progress of patients over time (Table 6.5) is shown with number of patients remaining in the study, the number of major amputations and survivor function with 95% confidence intervals at each time point of 1000, 2000, 3000 and 3500 days.

Table 6.5: Kaplan-Meier freedom from major amputation rate of cohort with 95% confidence intervals displayed by days follow up.

Major amputation		0 days	1000 days	2000 days	3000 days	3500 days
Total Cohort	At Risk	398	207	101	21	6
	Events in previous interval	0	20	5	2	0
	Freedom from major amputation (95% CI)	1	0.94 (0.91-0.97)	0.91 (0.87-0.95)	0.87 (0.80-0.94)	0.87 (0.80-0.94)

CI = Confidence Interval

Table 6.5 demonstrates that 74% of the 27 major amputations took place in the first 1000 days of the study. The number of patients remaining in the study and the number of events may be used to interpret the number of patients being discharged from the study at each time point of 1000, 2000, 3000 and 3500 days: giving 171, 101, 78 and 15 patients respectively. The survivor function and 95% confidence intervals do not change from 3000 to 3500 days as there were no major amputations in this period.

6.6. Cohort peripheral revascularisation Kaplan-Meier analysis

Kaplan-Meier survival plot of the freedom from peripheral revascularisation in the cohort is shown in Figure 6.7 and demonstrates that the cohort experienced this endpoint in a very different timeframe to the other outcomes. The sharp initial drop and concave shape of the freedom from peripheral revascularisation plot reveal that many of the patients underwent peripheral revascularisation early after recruitment to the study.

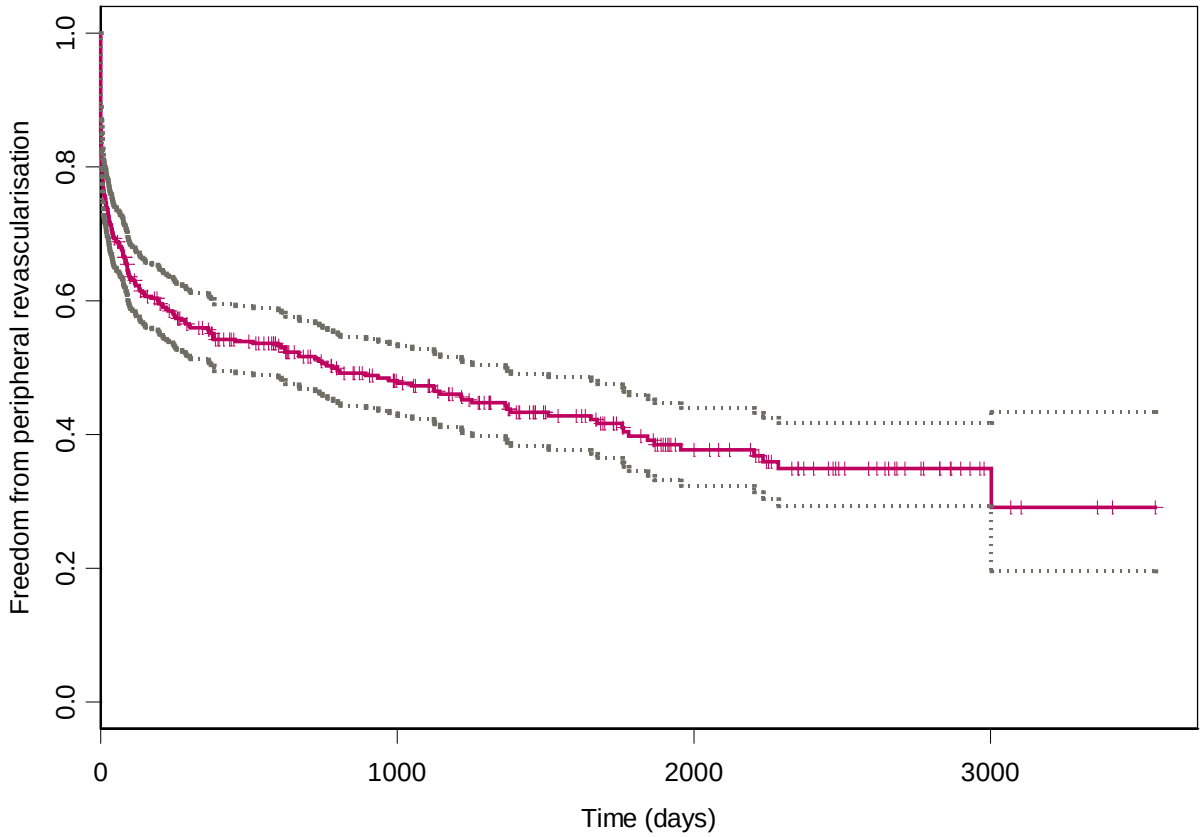

Figure 6.7: Kaplan-Meier non-parametric survival plot - freedom from peripheral revascularisation of cohort with 95% confidence intervals.

With 220 patients undergoing peripheral revascularisation during the course of the study the incidence of peripheral revascularisation was 55.3% with 178 patients discharged from follow up without having undergone a peripheral revascularisation procedure.

The calculated mean time to peripheral revascularisation was 1501 ± 90.7 days (4.1 years) but 50% of the population at risk had undergone peripheral revascularisation by day 782 (382 – 1364). The initial steepness of the curve (Figure 6.7) was caused by 174 patients undergoing peripheral revascularisation within the first year, while only 32 patients were discharged in that year with the freedom from peripheral revascularisation function almost at 50% by this time (55.4%). A large difference between median and mean can be seen in skewed data, with both median and mean together giving a better understanding of the data distribution. It takes another year for five percent of the population to require peripheral revascularisation showing the rapid change of freedom from peripheral revascularisation rate over this early course of the study.

The total of 295 633 patient follow up days is much less than previous endpoints and is largely made up of the population who did not undergo early revascularisation but remain in the study at risk thus elevating the calculated mean.

The progress of patients over time (Table 6.6) is shown with number of patients remaining in the study, the number of peripheral revascularisations and freedom from peripheral revascularisation function with 95% confidence intervals at each time point of 1000, 2000, 3000 and 3500 days.

Table 6.6: Kaplan-Meier freedom from peripheral revascularisation rate of cohort with 95% confidence intervals displayed by days follow up.

Peripheral Revascularisation		0 days	1000 days	2000 days	3000 days	3500 days
Total Cohort	At Risk	398	123	49	6	1
	Events in previous interval	0	197	19	3	1
	Freedom from peripheral revascularisation (95% CI)	1	0.48 (0.43-0.53)	0.38 (0.32-0.44)	0.35 (0.30-0.42)	0.29 (0.20-0.43)

CI = Confidence Interval

The progress of patients over time (Table 6.6) confirms that the freedom from peripheral revascularisation rate is <0.5 prior to 1000 days with 197 patients undergoing revascularisations prior to this point and 78 patients discharged from follow up in the same period. The further decline of the freedom from peripheral revascularisation rate can be seen with ongoing events to 3500 days. The step down in freedom from peripheral revascularisation rate at 3000 days (Figure 6.7) is the last peripheral revascularisation in the cohort during the study, with four other patients being discharged from follow up after 3000 days without requiring revascularisation. The last patient being followed was discharged at 3555 days without requiring revascularisation.

6.7. Discussion

The overall incidence of major adverse event during this study is similar to other populations of patients with peripheral arterial occlusive disease discussed in Chapter 3.[223] A total of 35.16% of the initially recruited population sustained a major adverse event with median freedom from major adverse event of 7.2 years. The observed average yearly rate of 4.9% major adverse event per year is consistent with Giugliano et al.[74] who reported an average 4.3% per year incidence of major adverse event over 30 month follow up of patients with peripheral arterial occlusive disease. Amaranto et al.[320] reported a lower average 3.7% yearly incidence of major adverse event in patients undergoing major open or endovascular procedures. Major adverse event in the ADEP (Atherosclerotic Disease Evolution by Picotamide) study[73] was less with 2.7% average incidence per annum despite their endpoint including major amputation. The incidence of major adverse events was greater in the PACK (Prevention of Atherosclerotic Complication with Ketanserin) study 20 years earlier, with Dormandy and Murray[24] reporting a 6.8% incidence of major adverse event, possibly reflecting the improved outcomes from medical and surgical management of atherosclerotic disease in the interval between these studies. This data confirms that the estimated rate of major adverse event rate used in power calculations (30%) was slightly conservative and the power calculations confirm adequate power for the study of this endpoint in this population.

Mortality rate of the studied population is similar to previously examined populations with peripheral arterial occlusive disease. The 100 deaths over the duration of the study occurred with mean freedom from death of 7.7 years. The total mortality rate over the duration of the study was 25.1% with an average yearly mortality rate of 3.3% which is comparable to the

average 3.8% yearly mortality reported by Arain et al.[222] although greater than the 1.1% average yearly mortality in the ADEP study[73] and the 1.7% average yearly mortality following major open and endovascular procedures reported by Amaranto et al.[320]. The average yearly mortality rate of this study was less than the 4.3% yearly claudicant mortality of Dormandy and Murray[24] and 4.8% first year mortality in the study of a similar population by Bloor[23]. The population of Bhutta et al.[49] was observed to have a higher average annual mortality of 6.1% per year in 1021 patients undergoing vascular surgery which may have been due to inclusion of a higher risk population or operative mortality being significantly different to the mortality of patients managed conservatively. This data confirms that the estimated mortality rate used in power calculations (25%) was very close to the observed rate (25.1%) and that power calculations confirm adequate power for the study of the endpoint of mortality in this population.

The incidence of heart attack in this study is similar to previously published studies. Heart attacks were sustained by 34 of the participants in this study with a mean freedom from heart attack of 9.0 years giving an average rate of 0.9% per year, identical to the average yearly occurrence of heart attack in the ADEP study[73]. Other populations with peripheral arterial occlusive disease exhibit higher rates of heart attack with the average rate of 2.2% heart attacks per year in the Giugliano et al.[74] population, average 2.1% per year in Haumer et al.[72] population and 1.8% in Dormandy and Murry[24] claudicant population. One possible cause for this lower rate in this study is methodological as a heart attack resulting in death was recorded as death and not heart attack.

Stroke occurred in this study with similar frequency to previously published studies. Mean freedom from stroke was 9.8 years and equated to an average rate of 0.46% stroke per year which is similar to the 0.6% average from the ADEP study[73]. Two previous studies have reported more than double this rate with an average of average of 1.1% per year in the Giugliano et al.[74] population and 1.4% in the Dormandy and Murray[24] claudicants. Haumer et al.[72] recorded a much higher average incidence of stroke of 3.1% per year although it is unclear how much of this difference could be accounted for by the inclusion of fatal strokes in this group.

The rate of major amputation in this study was between previously published rates of major amputation. Mean freedom from major amputation was 9.9 years with an average annual incidence of major amputation of 0.7% per year which was more than triple the 0.2% average major amputation rate per year in the ADEP study[73] although this study excluded Rest Pain and Tissue Loss patients who have an inherently higher rate of major amputation.[26,97] Interestingly the annual rate of major amputation was less than half of the 1.6% major amputation rate of Dormandy and Murray[24] study which only included patients with intermittent claudication.

The skewed nature of the freedom from peripheral revascularisation results of the cohort (Figure 6.7) was confirmed by the large difference in the median and mean freedom from peripheral revascularisation. This skewed data is largely a function of the described methods of this study which outlined the timing of patient recruitment into the study. Only patients with symptomatic peripheral arterial occlusive disease were included in this study and were recruited from the outpatient department or when they presented to hospital to undergo a

peripheral revascularisation procedure. Because this patient group was recruited at a time when they required peripheral revascularisation or were being considered for peripheral revascularisation, the time to peripheral revascularisation is understandably short in a large portion of the population thus providing the left skew to this endpoint. The peripheral revascularisation endpoint is not analysed in the following results chapters due to the skewed nature of the data distribution and the inherent bias due to the method of patient recruitment and inclusion criteria of this study.

6.8. Conclusion

The incidence of adverse cardiovascular outcomes in the studied patient group with symptomatic peripheral arterial occlusive disease is similar to previously studied populations with this disease. Some of the reported differences in the incidence of cardiovascular outcomes are due to the methodological differences between studies. The main methodological difference between this study and some previous studies is that heart attack that resulted in the patient death in this study was recorded as death, thus the outcome of heart attack in this study only includes non-fatal heart attacks. Similarly if the patient sustained a stroke that resulted in death then that was recorded as death making the outcome of stroke only the non-fatal strokes. Including the fatal events in the stroke and heart attack endpoints would have increased the event numbers of these outcomes and may have improved the power for statistical analysis to examine these endpoints individually. Future reporting of the number of the individual events within composite outcomes is important as it will allow detailed future comparisons and compilations for outcomes with comparatively small incidence in patients with peripheral arterial occlusive disease.

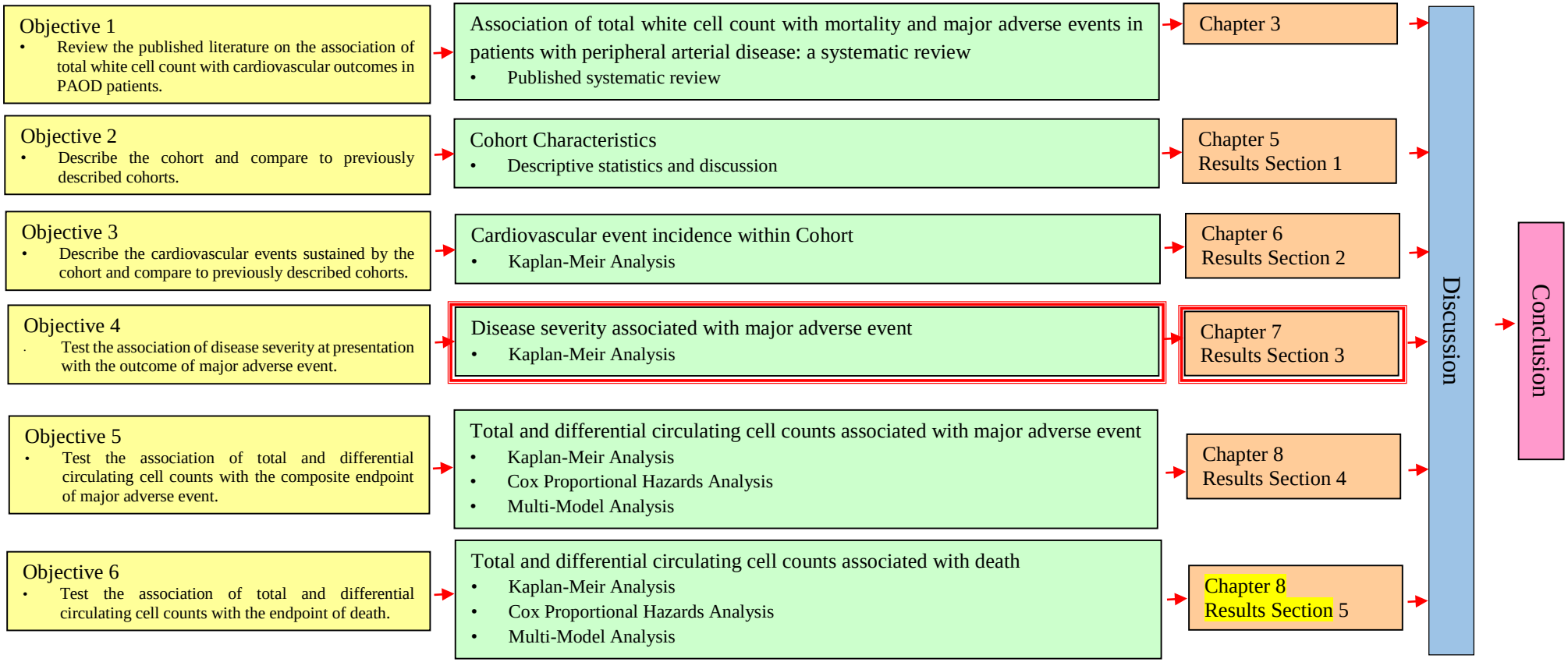

Figure 6.8: Schematic overview of thesis with red box highlighting current position within document – disease severity associated with major adverse event

7. Results - section 3

Disease severity associated with major adverse events in patients with peripheral arterial occlusive disease

7.1. Introduction

Disease severity of patients with peripheral arterial occlusive disease has been associated with cardiovascular events[8,24,26,28] however it has been inconsistently adjusted for in analysis of cardiovascular events in this population.[223] Patients with symptomatic peripheral arterial occlusive disease have a 15-year accrued survival rate of approximately 22%, compared with a survival rate of 78% in patients without peripheral arterial occlusive disease symptoms.[374] The San Diego Artery study[29] that followed patients with peripheral arterial occlusive disease over 10 years found that survival rates decreased with increasing severity of peripheral arterial occlusive disease symptoms.[29]

Patients with intermittent claudication are expected to have an annual major adverse event rate of 5%-7% which has been reported as being 2.5 times higher than age matched controls independent of risk factors.[28] Patients with critical limb ischaemia (rest pain or tissue loss) have a higher risk of sustaining cardiovascular ischaemic events than those with intermittent claudication[25], with a ~20% mortality in the first year after presentation[26,28,375] and mortality approaching 50% at 5 years and 70% at 10 years[27,28,376,377]. Conte et al.[378] developed objective performance goals to define appropriate outcome measures for critical limb ischaemia trials

and advocated the composite outcome of death, heart attack and stroke (among others) for investigating the outcomes of critical limb ischaemia with new and evolving treatments.

Major amputation has been previously observed in 12% of a large prospective multicentre cohort of patients with critical limb ischaemia at six months.[375] Following revascularisation with vein bypass, patients with critical limb ischaemia still have a 12% major amputation rate at one year.[379] If treated conservatively then as many as 38% of patients with critical limb ischaemia will require major amputation at one year with only 25% of ulcers healed at 12 months and 50% healed at one year.[108] Very different rates of major amputation are experienced by patients with intermittent claudication with between one and three percent[24,28] requiring major amputation per year.

Despite these apparent differences in outcomes based on disease severity, disease severity is not always reported or adjusted for in statistical analysis of outcome in patients with peripheral arterial occlusive disease.[223]

The hypothesis being tested in this chapter is that disease severity is associated with the outcomes of major adverse events (composite outcome of death, heart attack or stroke) in a population of patients with peripheral arterial occlusive disease.

7.2. Methods

The hypothesis of this study was tested through a prospective cohort study, the methodology is detailed in Chapter 4 with definitions of disease severity presented in Appendix I. The division of critical limb ischaemia into Rest Pain and Tissue Loss groups has been suggested

previously as these two groups have appreciably different natural histories.[26] To enable comparison with other literature the survival rates are also presented for 365 days. 3500 days is shown consistently throughout the results chapter as no patients remained in the study alive at 4000 days (10.96 years).

7.3. Results

The results of the entire cohort grouped by disease severity at presentation is examined with the endpoint of major adverse event. Kaplan-Meir survival analysis is presented through both graphical and tabular form to demonstrate timeline of events over the duration of the study and Log rank testing to assess statistical difference between the groups for each endpoint.

Kaplan-Meir plot of the freedom from composite endpoint of major adverse event (either death, heart attack or stroke) is shown in Figure 7.2 grouped by disease severity at presentation for inclusion into the study with the dotted lines representing the 95% confidence intervals. The horizontal axis is again displayed in days with the time points described in Chapter 4. The disease severity groups do not have equal numbers of patients and the survival rate plots need to be interpreted with consideration of the number of patients in that group. The Intermittent Claudication group (green, Figure 7.2) has the smoothest freedom from major adverse event curve because the largest number of patients (291) are in that group. The Acute Lower Limb Ischaemia group (orange, Figure 7.2) has the smallest number of patients (13) resulting in comparatively large drops in the freedom from major adverse event curve with each major adverse event.

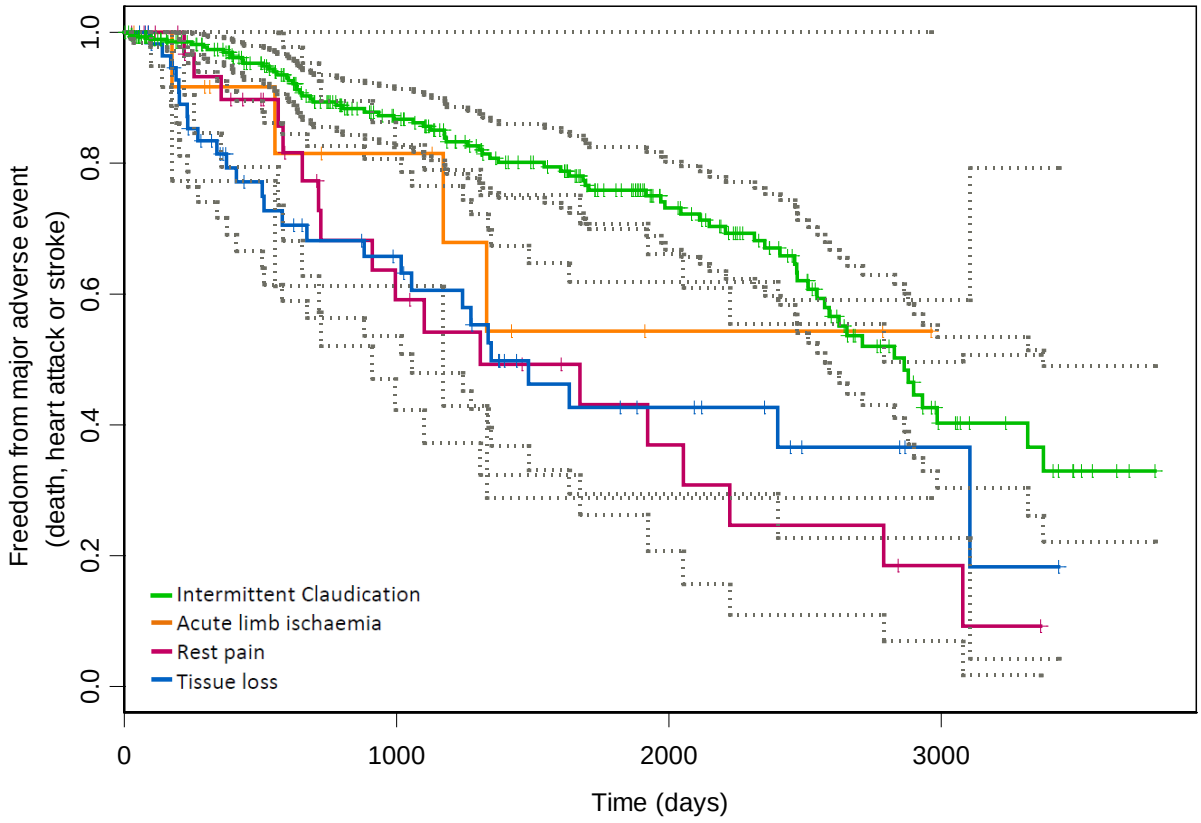

Figure 7.2: Kaplan-Meir non-parametric survival plot - freedom from major adverse event (death, heart attack or stroke) by disease severity at presentation with 95% confidence intervals.

The Rest Pain group (red, Figure 7.2) and Tissue Loss group (blue, Figure 7.2) follow a visibly different course to the Intermittent Claudication group (green, Figure 7.2) with a lower freedom from major adverse event for the majority of the study. The Tissue Loss plot initially takes a lower freedom from major adverse event course than the Rest Pain group until they meet at ~750 days and experience similar freedom from major adverse event rates until prior to 2000 days when the Rest Pain group falls lower and remains lower for the remainder of the study. The Acute Lower Limb Ischaemia group follows a course lower than that of the Intermittent Claudication group for most of the study but crosses plots at ~2750 days.

The Intermittent Claudication group sustained the largest number of major adverse events with 75 although this group had the largest number of patients (291) meaning 25.8% of the Intermittent Claudication group sustained a major adverse event over the course of the study. The Intermittent Claudication group had the longest calculated mean freedom from major adverse event of 2628 ± 95.3 days (7.20 years) and the longest median freedom from major adverse event 2864 (2572 – 3375) days. The shortest mean freedom from major adverse event was 1626 ± 219.5 days (4.45 years) in the Rest Pain group in which 35 patients (51.4% of that population) sustained a major adverse event. The Rest Pain group also had the shortest median freedom from major adverse event with 1308 (911 – 2789) days. Of the Tissue Loss group, 45.8% (27 of 59) sustained a major adverse event over the duration of the study with a mean freedom from major adverse event of 1775 ± 200.4 days. The median freedom from major adverse event of both the Tissue Loss group (1348 days = 3.69 years) and the Rest Pain group (1308 days = 3.58 years) are less than half of that for the Intermittent Claudication group (2864 days = 7.85 years). Within the Acute Lower Limb Ischaemia group, 30.8% (4 of 13) sustained a major adverse event over the duration of the study with mean freedom from major adverse event 2020 days (5.5 years) with the median not applicable for this group.

The average annual incidence of major adverse event over the entire duration of the study was 3.6% for the Intermittent Claudication group, 11.6% for the Rest Pain group, 9.4% for the Tissue Loss group and 5.6% for the Acute Lower Limb Ischaemia group. The observed rate of major adverse event was not consistent over time as can be seen in Figure 7.2 and Table 7.1.

The progress of patients over time is shown in Table 7.1 with the number of patients remaining in the study, the number of events and survivor function with 95% confidence intervals at each time point of 1000, 2000, 3000 and 3500 days.

Table 7.1: Kaplan-Meir freedom from major adverse event (death, heart attack or stroke) rate by disease severity at presentation with 95% confidence intervals displayed by days follow up.

Major adverse event		0 days	1000 days	2000 days	3000 days	3500 days
Intermittent Claudication	At Risk	291	159	80	16	5
	Events in previous interval	0	30	20	23	2
	Freedom from major adverse event (95% CI)	1.00	0.87 (0.82-0.94)	0.73 (0.67-0.80)	0.40 (0.30-0.53)	0.32 (0.22-0.49)
Acute Lower Limb Ischaemia	At Risk	13	7	3	-	-
	Events in previous interval	0	2	2	-	-
	Freedom from major adverse event (95% CI)	1.00	0.82 (0.61-1.00)	0.54 (0.23-1.00)	-	-
Rest Pain	At Risk	35	13	6	2	-
	Events in previous interval	0	10	4	3	-
	Freedom from major adverse event (95% CI)	1.00	0.59 (0.42-0.83)	0.37 (0.21-0.66)	0.19 (0.07 0.50)	-
Tissue Loss	At Risk	59	26	10	2	-
	Events in previous interval	0	17	8	1	-
	Freedom from major adverse event (95% CI)	1.00	0.66 (0.54-0.81)	0.43 (0.29-0.62)	0.37 (0.23-0.59)	-

CI = Confidence Interval

In the group of 291 patients that presented with Intermittent Claudication, 30 patients sustained a major adverse event in the first 1000 days, with 102 being discharged from follow up in this time (Table 7.1). From 1000 to 2000 days a further 20 patients had a major adverse event and 59 patients were discharged. Between 2000 and 3000 days 23 patients from the Intermittent Claudication group had a major adverse event and 41 patients were discharged from follow up. In the period from 3000 to 3500 days two further patients had major adverse events while nine

patients were discharged. Between 3500 follow up days and the conclusion of the study there were no further major adverse events in the remainder of the group and those five patients were discharged from follow up without event (Table 7.1).

The Acute Lower Limb Ischaemia group of 13 patients sustained four major adverse events over the course of the study, two of these events were prior to 1000 days and two events occurred between 1000 and 2000 days (Table 7.1). The discharge from follow up was four patients prior to 1000 days, two patients between 1000 and 2000 days and the remaining three between 2000 and 3000 days (Table 7.1).

Ten of the 35 patients with Rest Pain had a major adverse event prior to 1000 days with 12 patients discharged from follow up in this time (Table 7.1). Between 1000 and 2000 days there were four major adverse events in the Rest Pain group with three patients discharged from follow up. From 2000 to 3000 days there were a further three major adverse events with one patient discharged from follow up. The last two remaining patients in the Rest Pain group were discharged prior to 3500 days (Table 7.1).

Of the 59 patients in the Tissue Loss group, 17 sustained a major adverse event prior to 1000 days with 16 patients from this group discharged in that time (Table 7.1). Between 1000 and 2000 days there were a further eight major adverse events and eight patients discharged from follow up. The last event occurred in the 2000 to 3000 day window, with seven patients discharged from follow up. No patients who presented with Tissue Loss remained in the study at 3500 days (Table 7.1).

At one year the Tissue Loss group freedom from major adverse event function is 0.81 (95% CI 0.72 – 1.00) having sustained 10 major adverse events in the first 365 days of the study. The freedom from major adverse event function of the Rest Pain group at 365 days is 0.90 (95% CI 0.79 -1.00) having sustained three major adverse events in the first year of the study. The Acute Lower Limb Ischaemia group had one major adverse event in the first year with resultant freedom from major adverse event rate 0.92 (95% CI 0.77 – 1.00). There were eight major adverse events in the Intermittent Claudication group within the first year giving this group a freedom from major adverse event function of 0.97 (95% CI 0.95 – 0.99).

Kaplan-Meier freedom from death function presented in both graphical (Figure 7.2) and tabular (Table 7.1) form indicate that the Intermittent Claudication group is distinct from both Rest Pain and Tissue Loss groups for the endpoint of major adverse event. A log rank test was performed to test the hypothesis that there is no difference between these survival curves for major adverse event. The purpose of the log rank analysis was to test if disease severity at presentation was a potential confounding variable for major adverse event that should be adjusted for when testing the association between circulating cell counts and cardiovascular outcomes in Chapter 8 Section 4 and 5. The log rank test resulted in a Chi-square value of 26.1 and a p-value <0.01 (probability of obtaining a test statistic as extreme as this if there is no difference in survival curves). This high degree of significance on the log rank test indicates that there may be a true difference in the survival curves between the disease severity groups however it does not attribute this risk to the disease severity alone as there has not been adjustment for confounding factors.

7.4. Discussion

Disease severity at presentation in patients with peripheral arterial occlusive disease has significant association with the cardiovascular endpoint of major adverse event. The contribution of disease severity to outcome should be considered and statistically adjusted for when assessing other factors that may be associated with cardiovascular outcomes in patients with peripheral arterial occlusive disease.

Disease severity at presentation groups were significantly different on log rank testing ($p<0.01$) for the outcome of major adverse event. The Intermittent Claudication group has the lowest incidence of major adverse event with 25.8%, the average annual rate of major adverse event in the Intermittent Claudication population was 3.6% over the duration of the study although in the first year of the study it was only 3%. This population is below the expected range reported by TASC II who reported the annual overall rate of major adverse event to be approximately 5-7% per year.[28] Intermittent claudicants in the PACK (Prevention of Atherosclerotic Complication with Ketanserin) study were at the other end of this range with an annual rate of 6.8% major adverse events reported by Dormandy and Murray.[24] The Tissue Loss group initially has the lowest freedom from major adverse event function (0.81 at one year) with 19% of that group sustaining a major adverse event in the first year. However, the Rest Pain freedom from major adverse event function drops below the Tissue Loss group prior to 1000 days. When the groups of Rest Pain and Tissue Loss are combined into a single group of Critical Limb Ischaemia, the total incidence of major adverse event in this population is 47.9% over the entire duration of the study. The composite endpoint of major adverse event needs further research in the subgroup of patients with Critical Limb Ischaemia as recommended by Conte et al.[378]

Patients with critical limb ischaemia often have their risk factors for atherosclerotic disease inadequately treated compared with patients with ischaemic heart disease or cerebrovascular disease.[127,380,381] This is a potential contributing factor to the worse outcomes of this sub population in this study with less aspirin use in Rest Pain (65.7%) and Tissue Loss (62.7%) group patients than in the population of Intermittent claudication (71.1%, Table 5.1) and only 8.5/8.6% of the Rest Pain and Tissue loss groups taking another antiplatelet agent compared to 15.5% of the Intermittent Claudication group. Statin use however was proportionately higher in the Rest Pain group (71.4%) compared to the Tissue Loss group (62.7%) and Intermittent Claudication group (64.9%, Table 5.1).

The higher incidence of major adverse events in the disease severity groups of Rest Pain and Tissue loss are possibly an indicator of the burden of systemic atherosclerotic disease, with greater burden in both the coronary, cerebral as well as peripheral circulation. The ischaemia of tissue (in this case in the lower limb) and has previously been shown to affect white blood cell adhesion and deformability resulting in adverse effects in remote organs.[230,231,341]

7.5. Conclusion

Disease severity is associated with the outcome of major adverse event. These results were comparable to other populations of peripheral arterial occlusive disease and demonstrate that disease severity should be reported and adjusted for in the analysis of outcomes for this population.

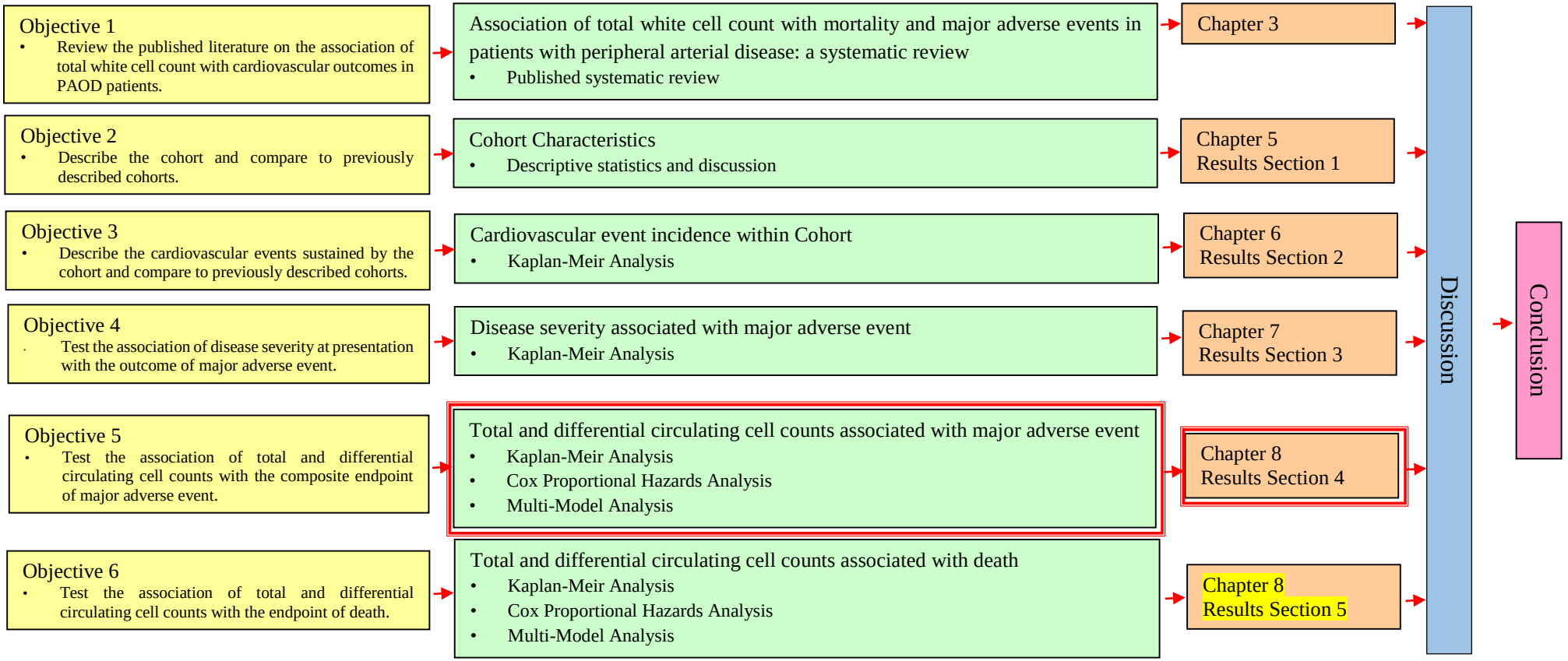

Figure 8.1: Schematic overview of thesis with red box highlighting current position within document –Circulating cell counts associated with major adverse event

8. Results - section 4 and 5

Total and differential circulating cell counts associated with major adverse events and death in patients with peripheral arterial occlusive disease

8.1. Introduction

Circulating cell counts of patients with peripheral arterial occlusive disease have been associated with major adverse events[24,49,69,72,99,222,260,266,320] and death.[24,49,72,74,99,122,152,222,238,260,316,321,322] Relative leucocytosis at admission for cardiovascular or cerebrovascular events correlates with severity of ischaemic damage and subsequent course,[99] and is associated with all-cause mortality in patients with peripheral arterial occlusive disease[24,222] and the subset with critical limb ischaemia.[122,322] Elevated total white cell count (TWCC) has also been associated with mortality after cardiac surgery[209] and percutaneous coronary intervention[205]. The relationship of total white cell count with the composite outcome of major adverse event may not always be linear, with previous report of a U shaped correlation after open operations for arterial disease.[320]

When analysing the total white cell count as subtypes, a neutrophil count of greater than 5.8 $x10^9$cells/L has previously been shown to be associated with an increased risk for major adverse events in patients with peripheral occlusive arterial disease diagnosed with >50% stenosis on angiogram.[72] The neutrophil count was the parameter that contributed most strongly

to increased risk of death in Grau et al.[69], and was a predictive index, particularly for vascular death (relative risk 1.86). Neutrophil count was also associated with death in Haumer et al.[72] study of 398 patients with symptomatic peripheral arterial occlusive disease.

High neutrophil count has also been shown to add prognostic information to traditional atherothrombotic risk factors in a population of 368 patients, 27% of whom had critical limb ischaemia although the hazard or risk ratios were not reported.[72]

Lymphocyte count demonstrated an inverse relationship with recurrent ischaemic events in the Clopidogrel versus aspirin in patients at risk of adverse ischaemic events trial (CAPRIE) of patients with heart attack, stroke and peripheral arterial occlusive disease.[69] This relationship has been replicated in large studies of patients with coronary artery disease following coronary percutaneous intervention,[205] and studies of left ventricular dysfunction.[208] To the authors knowledge there is no published evidence of lymphocyte count and the association with mortality in patients with peripheral arterial occlusive disease.

Monocyte count has previously been associated with the presence of peripheral arterial occlusive disease in population studies.[264] Monocyte count in patients with coronary artery disease is associated with adverse cardiovascular event although to a lesser extent than neutrophil count.[99] In patients with peripheral arterial occlusive disease circulating monocyte count has been associated with vein graft stenosis following lower limb bypass surgery but not mortality.[266] Evidence of any association of monocyte count with the composite endpoint of major adverse event remains to be demonstrated.

Low haemoglobin concentration (anaemia) may be an independent risk factor of cardiovascular risk in the general population[270] with the Framingham study demonstrating a U-shaped relationship between haematocrit and mortality.[271,272] Even mild anaemia has been shown (via haematocrit) in a very large population (310311 participants) to increase 30 day post-operative mortality and cardiac events in older male patients undergoing non-cardiac surgery.[287] In patients with coronary artery disease haemoglobin has been associated with early and late mortality[382] death following heart attack,[275] cardiac surgery,[276-278,280] percutaneous intervention,[215,272,281,282] and heart failure.[270,284] In patients undergoing elective vascular surgery low haemoglobin concentration has been shown to be associated with composite endpoint of death or heart attack with 30 day hazard ratio of 4.7 (2.6-10.9) for the severe anaemia group (haemoglobin 98±8 $x10^9$g/L).[288] In the same patient group the five year hazard ratio for heart attack or death was 6.1 (95% CI 4.1-9.1) adjusted for other comorbidities including renal function and heart failure.[288] Pre-operative haemoglobin has been associated with two year mortality in patients undergoing major vascular surgery,[49] and haematocrit was lower in patients who died during follow up for critical limb ischaemia.[322]

Although haemoglobin concentration has been associated with adverse surgical outcome in patients with peripheral arterial occlusive disease through vascular surgical incisional complications,[225] amputation site healing[224] and early loss of bypass patency,[289] the relationship between haemoglobin with mortality and major adverse events remains unclear in patients with peripheral arterial occlusive disease.

Pre-operative neutrophil-lymphocyte ratio (NLR) identified patients at increased risk of death following major vascular surgery[49] and in patients with chronic critical limb ischaemia.[260] In patients with coronary artery disease neutrophil-lymphocyte ratio has a strong relationship to adverse outcomes[229,242-245] in patients undergoing percutaneous coronary interventions[212,249] and coronary artery bypass grafting.[250,251]

The aim of this study was to combine the assessment of all the circulating cell types discussed above on the same population with peripheral arterial occlusive disease for the composite endpoint of major adverse event and death alone. A secondary aim is to generate a model that combines the cell count values (from an inexpensive, reliable data source that is already routinely obtained with known risk factors) as a predictive tool for major adverse event that may be applied by the clinician at the bedside. Developing the understanding of the true relationships and relative predictive value of the circulating cells combined with and compared to the known risk factors for major adverse event and death will not only better guide risk stratification models for patient treatment but guide further research into understanding of the pathogenesis of major adverse events and death in this population.

The hypothesis being tested in this study is that baseline total and differential circulating cell counts are associated with major adverse events (composite outcome of death, heart attack or stroke) and death alone in patients with peripheral arterial occlusive disease. The circulating cell counts investigated in this study are total white cell count (TWCC), neutrophil count, lymphocyte count, monocyte count, calculated neutrophil-lymphocyte ratio plus haemoglobin concentration as a measure of red blood cell parameters.

8.2. Methods

The two separate hypothesis to be tested in this chapter will be separately addressed in Section 4 for the outcome of major adverse event and Section 5 for the outcome of death. Both hypothesis were tested through a prospective cohort study, with the detailed methodology available in Chapter 4. Both hypothesis were tested by Kaplan-Meier non-parametric survival analysis, Cox proportional hazards analysis and multi-model averaging. Each section will be followed by a discussion and conclusion for that endpoint.

Kaplan-Meier analysis of freedom from major adverse event for the entire cohort grouped by cell count (or haemoglobin concentration) tertile is presented in both graphical and tabular form with the x axis time points described in Chapter 4 to show progress of patients over time throughout the study. Patients that were discharged from follow up without event are discussed.

Cox proportional hazards analysis for the outcome of major adverse event and death is presented for each circulating cell type *a priori*, adjusted for traditional risk factors (defined in Chapter 4) and adjusted for comprehensive risk factors (defined in Chapter 4) with the model stratified when required. Conformity of all Cox models with the assumptions of proportionality is tested using the Cox.zph score for each variable and each global model, any significant Cox.zph scores indicating a potential breach in the assumption of proportionality are discussed.

The multi-model averaging process is used to assess which models and subsequently which variables are best associated with the outcome of major adverse event and death separately.

Multi-model averaging included the comprehensive risk factors noted above and neutrophil category, lymphocyte category, monocyte category, haemoglobin category and calculated neutrophil/lymphocyte ratio category. Total white cell count category was unable to be used as it is an additive expression of the other cell types. The best models are presented with AICc and Akaike weight and described with relative variable importance for the outcome of major adverse event. The full model for each outcome that combines the most important variables with averaged coefficients is presented and the implications discussed.

8.3. Results section 4

Total and differential circulating cell counts associated with major adverse events in patients with peripheral arterial occlusive disease

Each circulating cell type is examined and presented in turn for association with the end point of major adverse event with Kaplan-Meier survival analysis and Cox proportional hazards analysis with and without adjustment for traditional and comprehensive confounding factors. The multi-model averaging process is presented at the end of this chapter to assess all cell types and comprehensive confounding factors together in modelling the outcome of major adverse event and will report which models and subsequently which variables are best associated with the outcome of major adverse event.

8.3.1. Total white cell count associated with major adverse event (death, heart attack or stroke)

8.3.1.1. Kaplan-Meier survival analysis

Kaplan-Meier freedom from major adverse event plot of the cohort is presented in Figure 8.2 by total white cell count (TWCC) category. The horizontal axis is displayed in days with time points described in Chapter 4. The cohort was divided into tertiles based on the "baseline bloods" TWCC using the quantile formula in S+. This resulted in the low tertile "Low TWCC Category" including baseline total white cell counts from 3.6 to <6.9 ($x10^9$ cells/L), the mid tertile "Mid TWCC Category" including baseline total white cell counts from 6.9 to <8.7 ($x10^9$ cells/L), and the upper tertile "High TWCC Category" including baseline total white cell counts

from 8.7 to 14.5 (x10^9 cells/L). There were no missing values for the continuous TWCC variable. The TWCC categories created remain consistent throughout all the results chapters.

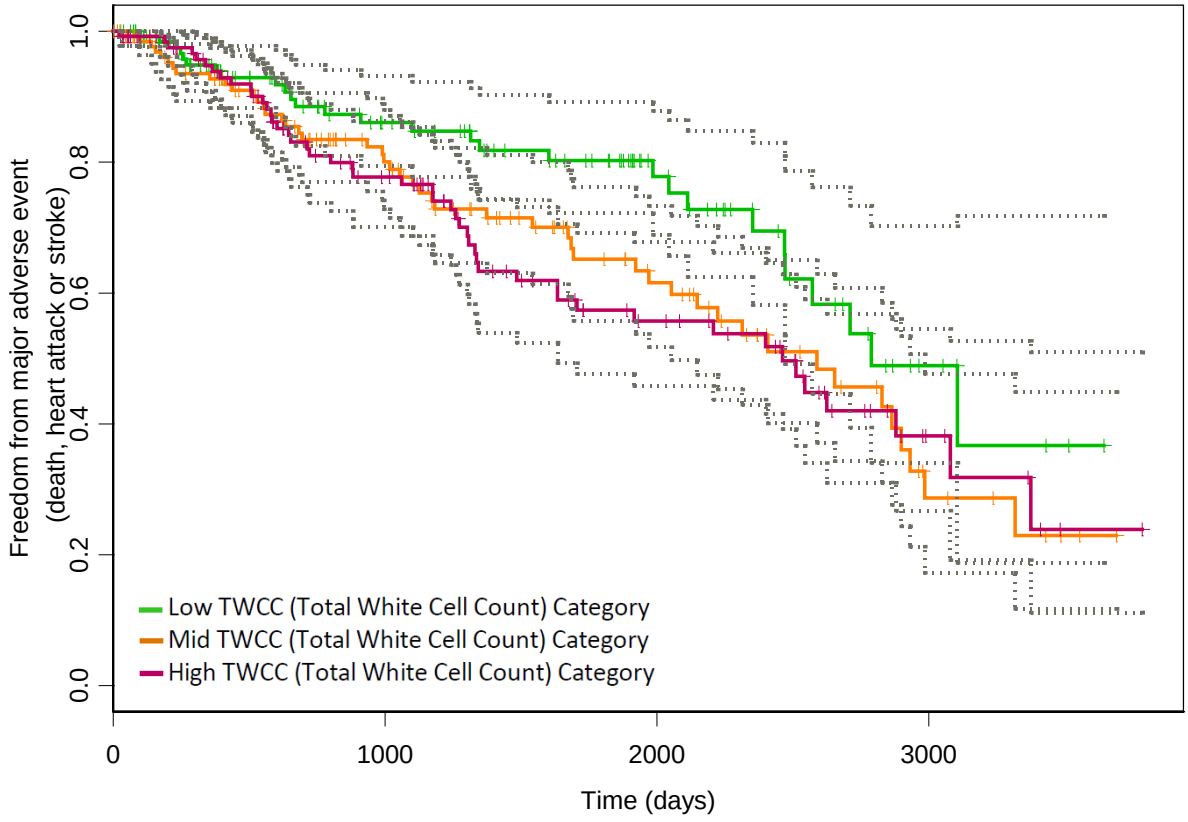

Figure 8.2: Kaplan-Meier non-parametric survival plot - freedom from major adverse event (death, heart attack or stroke) by total white cell count category with 95% confidence intervals

The freedom from major adverse event plot of the low TWCC group (green, Figure 8.2) appears visually distinct from the other two plots in Figure 8.2. There are multiple intersections between the Mid TWCC Category (orange, Figure 8.2) and High TWCC Category (red, Figure 8.2) freedom from major adverse event plots for the first 500 days then again at 1000 days, ~2500 days and prior to 3000 days.

The Low TWCC Category has the least number of major adverse events (28, 21.4% of the patients in this category) and has the greatest median (2789 days = 7.6 years) and mean (2631 ± 145 days = 7.2 years) freedom from major adverse events. The number of major adverse events that occurred in the High TWCC Category (48, 36.6% of the patients in this category) was the same as the Mid TWCC Category (48, 35.3% of the patients in this category) but the High TWCC Category experienced the shortest mean (2244 ± 146 days = 6.1 years) and median (2462 days = 6.7 years) freedom from major adverse events times. The Mid TWCC Category had a mean freedom from major adverse event of 2280 ± 133 days and median 2589 (2053 – 2985) days.

The progress of patients over time is shown in Table 8.1 with the number of patients remaining in the study at 1000, 2000, 3000 and 3500 days.

Table 8.1: Kaplan-Meier freedom from major adverse event (death, heart attack or stroke) rate by total white cell count category with 95% confidence intervals displayed by days follow up.

Major adverse event		0 days	1000 days	2000 days	3000 days	3500 days
Low TWCC Category	At Risk	131	66	32	6	2
	Events in previous interval	0	14	5	8	1
	Freedom from major adverse event (95% CI)	1.00	0.87 (0.79-0.93)	0.78 (0.69-0.88)	0.49 (0.34-0.70)	0.37 (0.19-0.72)
Mid TWCC Category	At Risk	136	70	34	7	2
	Events in previous interval	0	22	13	12	1
	Freedom from major adverse event (95% CI)	1.00	0.80 (0.73-0.88)	0.62 (0.52-0.73)	0.29 (0.17-0.48)	0.23 (0.18-0.45)
High TWCC Category	At Risk	131	69	32	7	1
	Events in previous interval	0	23	16	7	2
	Freedom from major adverse event (95% CI)	1.00	0.78 (0.70-0.86)	0.56 (0.46-0.68)	0.38 (0.27-0.55)	0.24 (0.11-0.51)

CI = Confidence Interval TWCC = Total White cell count

The Low TWCC Category has the greatest freedom from major adverse event rate at each of the reported time points (Table 8.1). The High TWCC Category has the lowest freedom from major adverse event rate for the 1000 day and 2000 day time points but the Mid TWCC Category has the lowest freedom from major adverse event rate at the 3000 and 3500 day time points (Table 8.1).

Apparent differences in the TWCC categories for the outcome of major adverse event with Kaplan-Meir analysis were then analysed with Cox proportional hazards model testing to allow adjustment for traditional and comprehensive risk factors and can be seen in Table 8.2.

8.3.1.2. Cox proportional hazards

Table 8.2: Cox proportional hazards analysis - major adverse event and total white cell count category.

Outcome - Major adverse event						
Cell Type		p Value	HR (95% CI)	Other Significant Variables	Cox zph	Global zph
TWCC *a priori*	mid	**0.05**	**1.60 (1.00-2.54)**	n/a	0.89	0.83
	high	**0.03**	**1.68 (1.06-2.69)**		0.59	
TWCC adjusted TRF[1]	mid	0.11	1.48 (0.92-2.37)	Age (p<0.01), IHD (p=0.02)	0.73	0.21
	high	**0.03**	**1.71 (1.06-2.77)**		0.81	
TWCC adjusted TRF[1] strata IHD	mid	0.11	1.48 (0.92-2.37)	Age (p<0.01)	0.73	0.23
	high	**0.02**	**1.75 (1.08-2.83)**		0.94	
TWCC adjusted CRF[2]	mid	**0.01**	**1.89 (1.15-3.09)**	Age (p<0.01), IHD (p<0.01), HTN (p=0.02), Ex-smoker (p=0.03), Smoker (p=0.07), Gender (p=0.08)	0.72	0.29
	high	**0.02**	**1.83 (1.12-2.99)**		0.76	
TWCC adjusted CRF[2] strata (Gender, smoking, HTN, IHD)	mid	**0.02**	**1.87 (1.10-3.18)**	Age (p<0.01)	0.79	0.98
	high	**<0.01**	**2.07 (1.21-3.54)**		0.63	

CI = Confidence Interval TWCC = Total white cell count n/a = not applicable
IHD = Ischaemic heart disease HTN = Hypertension HR = Hazard Ratio
TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
strata = statistical stratification for (variable/s in brackets following)
Significant p values at the 0.05 level and corresponding hazard ratio are presented in **bold**

The High TWCC Category is significantly associated with the endpoint of major adverse event in all models at the p<0.05 level when tested with the Cox proportional hazards analysis (Table 8.2). When the model was adjusted for traditional risk factors alone (adjusted TRF[1]) High TWCC Category remained significantly associated with major adverse events (p<0.05, Table 8.2) and when this analysis was stratified for the other significant categorical variable ischaemic heart disease High TWCC Category remained significantly associated with major adverse event (p=0.02, Table 8.2). When the model was adjusted for traditional risk factors, disease severity and medication use (adjusted CRF[2]) the High TWCC category was significantly associated with major adverse event (p=0.02, Table 8.2). This association was

persistently significant when the adjusted CRF^2 model was stratified for other significant categorical variables of ischaemic heart disease, hypertension, smoking and gender ($p<0.01$, Table 8.2). The Hazard ratio of 2.07 in the stratified model adjusted for comprehensive risk factors (adjusted CRF^2 stratified for Gender, smoking, HTN, IHD), Table 8.2) indicates that the High TWCC category is twice as likely as the Low TWCC Category to sustain a major adverse event when all other risk factors are taken into consideration.

The Mid TWCC Category is significantly associated with the endpoint of major adverse event at the $p=0.05$ level when tested with the Cox proportional hazards analysis *a priori* (Table 8.2). When the model was adjusted for traditional risk factors alone (adjusted TRF^1) Mid TWCC Category was not significantly associated with major adverse events or when this analysis was stratified for the other significant categorical variable ischaemic heart disease. When the model was adjusted for comprehensive risk factors: traditional risk factors, disease severity and aspirin and statin use (adjusted CRF^2), the Mid TWCC category was significantly associated with major adverse event ($p=0.01$, Table 8.2). This association was persistently significant when the adjusted CRF^2 model was stratified for other significant categorical variables of ischaemic heart disease, hypertension, smoking and gender ($p=0.02$, Table 8.2). The Hazard ratio of 1.87 in the stratified model adjusted for comprehensive risk factors (adjusted CRF^2 stratified for Gender, smoking, HTN, IHD), Table 8.2) indicates that the Mid TWCC category is almost twice as likely as the Low TWCC Category to sustain a major adverse event when all other risk factors are taken into consideration.

The probability of a violation of the assumptions of the Cox proportional hazards model was checked with the Cox.zph function for each model. The Cox.zph function tests the proportionality of each predictor in the model by creating interactions with time and comparing the residuals, in this case giving values 0.21 - 0.98, (Table 8.2) or a low probability of assumption violation for each of the TWCC and major adverse event models. With a low probability of assumption breach the Cox proportional hazards models are appropriate to use for this data set and generate valid results.

8.3.2. Neutrophil count associated with major adverse event (death, heart attack or stroke).

8.3.2.1. Kaplan-Meier survival analysis

Kaplan-Meier freedom from major adverse event plot of the cohort is presented in Figure 8.3 by neutrophil count category. The cohort was divided into categories of neutrophil count with the quantile formula in S+. This resulted in the Low Neutrophil Category including baseline neutrophil counts from 1.4 to <4.0 ($x10^9$ cells/L), the Mid Neutrophil Category from 4.0 to <5.5 ($x10^9$ cells/L), and the High Neutrophil Category from 5.5 to 12.75 ($x10^9$ cells/L). Categorisation of the neutrophil data was done to minimise non-linear effects of the variable on the outcome. There were no missing values for the continuous neutrophil variable. These categories remain consistent throughout all the results chapters.

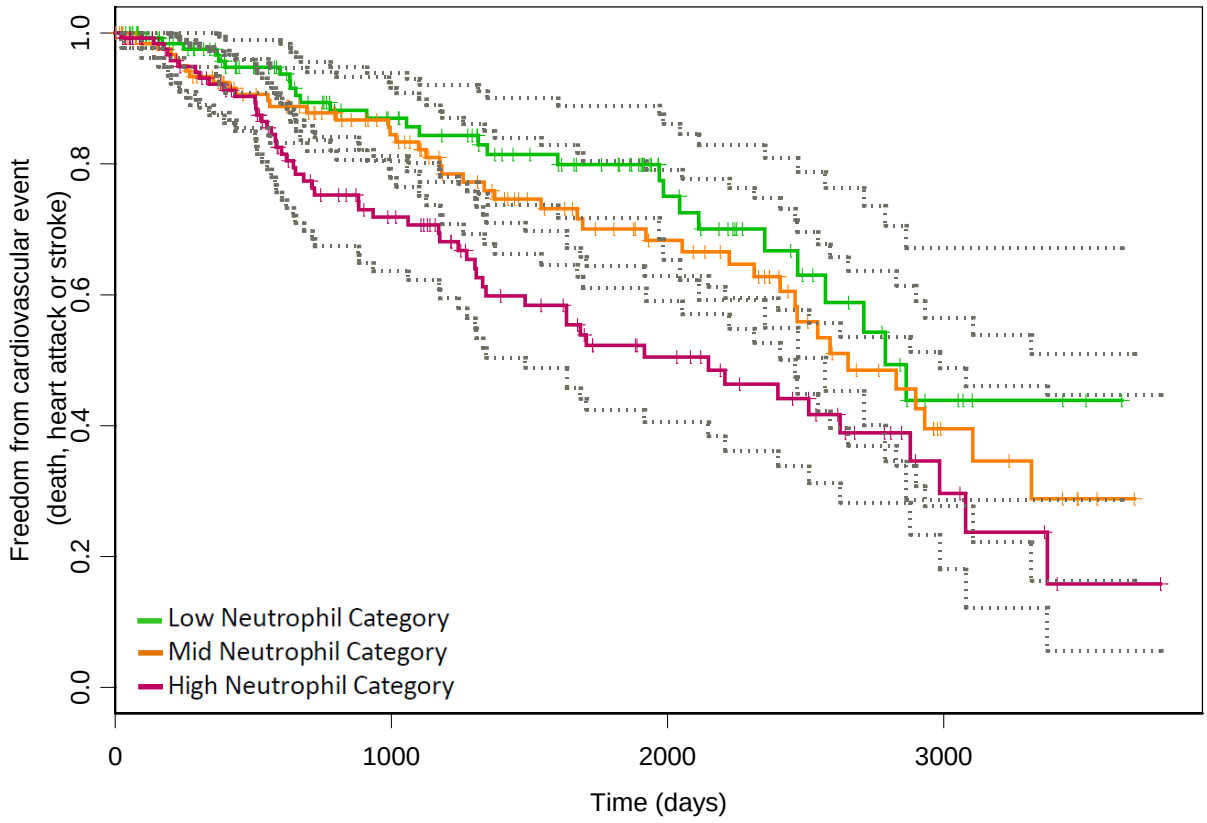

Figure 8.3: Kaplan-Meier non-parametric survival plot - freedom from major adverse event (death, heart attack or stroke) by neutrophil count category with 95% confidence intervals.

In Figure 8.3 the three neutrophil categories appear to have mostly distinct rates of freedom from major adverse event over the course of the study. The High Neutrophil Category (red, Figure 8.3) appears visually to be separate from the other two plots for Low and Mid Neutrophil Category for the outcome of major adverse event despite a similar trajectory to the Mid Neutrophil Category <500 days. Whilst there is a trend to separation of the Low Neutrophil Category (green, Figure 8.3) from the Mid Neutrophil Category (orange, Figure 8.3) there is an intersection of the freedom of major adverse event plots prior to 3000 days.

The High Neutrophil Category experienced the greatest number of major adverse events (53, 40.8% of the patients in this category) over the course of the study with the lowest mean (2051 ± 143 days = 5.6 years) and median of 2148 days (1484 – 2985) or 5.9 years freedom from major adverse event. The Low Neutrophil Category has the lowest number of major adverse events (28, 20.6% of the patients in this category) over the course of the study and has the greatest mean (2655 ± 143 days = 7.3 years) and median (2789 days = 7.6 years) freedom from major adverse event. The Mid Neutrophil Category experienced 43 major adverse events in 132 patients, with mean freedom from major adverse event 2447 ± 132 days (6.7 years) and median 2654 days or 7.3 years.

Table 8.3: Kaplan-Meier freedom from major adverse event (death, heart attack or stroke) rate by neutrophil count category with 95% confidence intervals displayed by days follow up.

Major adverse event		0 days	1000 days	2000 days	3000 days	3500 days
Low Neutrophil Category	At Risk	136	68	31	6	2
	Events in previous interval	0	13	7	8	0
	Freedom from major adverse event (95% CI)	1.00	0.87 (0.81-0.94)	0.75 (0.65-0.86)	0.44 (0.29-0.67)	0.44 (0.29-0.67)
Mid Neutrophil Category	At Risk	132	75	39	8	2
	Events in previous interval	0	17	12	12	2
	Freedom from major adverse event (95% CI)	1.00	0.85 (0.78-0.92)	0.68 (0.59-0.79)	0.40 (0.28-0.57)	0.29 (0.16-0.51)
High Neutrophil Category	At Risk	130	62	28	6	1
	Events in previous interval	0	29	15	7	2
	Freedom from major adverse event (95% CI)	1.00	0.72 (0.64-0.81)	0.52 (0.42-0.63)	0.30 (0.18-0.49)	0.16 (0.06-0.45)

CI = Confidence Interval

The High Neutrophil Category has the greatest number of major adverse events (29) prior to 1000 days and has the lowest freedom from major adverse events at all reported time points (Table 8.3). The Low Neutrophil Category has the greatest freedom from major adverse events at all reported time points (Table 8.3).

Apparent differences in the neutrophil categories for the outcome of major adverse event with Kaplan-Meir analysis were then analysed with Cox proportional hazards model testing to allow adjustment for traditional and comprehensive risk factors and can be seen in Table 8.4.

8.3.2.2. Cox proportional hazards

Table 8.4: Cox proportional hazards analysis - major adverse event and neutrophil category

Outcome - major adverse event						
Cell Type		p Value	HR (95% CI)	Other Significant Variables	Cox zph	Global zph
Neutrophil *a priori*	mid	0.23	1.34 (0.83-2.16)	n/a	0.81	0.64
	high	**<0.01**	**2.06 (1.30-3.26)**		0.39	
Neutrophil adjusted TRF[1]	mid	0.28	1.31 (0.81-2.13)	Age (p<0.01), IHD (p=0.04)	0.56	0.22
	high	**<0.01**	**1.94 (1.21-3.10)**		0.57	
Neutrophil adjusted TRF[1] strata IHD	mid	0.25	1.33 (0.82-2.17)	Age (p<0.01)	0.62	0.23
	high	**<0.01**	**1.95 (1.22-3.13)**		0.60	
Neutrophil adjusted CRF[2]	mid	0.15	1.44 (0.88-2.35)	Age (p<0.01), Ex-smoker (p=0.02), HTN (p=0.02), IHD (p=0.02), Smoker (p=0.04)	0.97	0.25
	high	**<0.01**	**2.02 (1.24-3.27)**		0.92	
Neutrophil adjusted CRF[2] strata (smoking, IHD & HTN)	mid	0.11	1.35 (0.91-2.54)	Age (p<0.01)	0.72	0.88
	high	**0.02**	**1.98 (1.12-3.10)**		0.75	

CI = Confidence Interval n/a = not applicable
IHD = Ischaemic heart disease HTN = Hypertension
TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
Significant p values at the 0.05 level and corresponding hazard ratio are presented in **bold**

The High Neutrophil Category is significantly associated with the endpoint of major adverse event in all models at the p<0.05 level when tested with the Cox proportional hazards analysis (Table 8.4). In the *a priori* model High Neutrophil category was associated with major adverse events at the p<0.01 level (Table 8.4) with a hazard ratio of >2 indicating that without adjusting for other risk factors the High Neutrophil Category was twice as likely to sustain a major adverse event than the Low Neutrophil Category. When the model was adjusted for traditional risk factors alone (adjusted TRF[1]), High Neutrophil Category remained significantly associated with major adverse events (p<0.01, Table 8.4) and when this analysis was stratified for the other significant categorical variable ischaemic heart disease, High Neutrophil Category

remained significantly associated with major adverse event ($p<0.01$, Table 8.4) although the hazard ratio dropped slightly below 2 in both these models. When the model was adjusted for traditional risk factors, disease severity and medication use (adjusted CRF^2) the High Neutrophil category was significantly associated with major adverse event ($p<0.01$, Table 8.4). This association was persistently significant when the adjusted CRF^2 model was stratified for other significant categorical variables of ischaemic heart disease, hypertension, smoking and gender ($p=0.02$, Table 8.4) with the hazard ratio approaching 2 meaning that the High Neutrophil Category was almost twice as likely to sustain a major adverse event as the Low Neutrophil Category.

The Mid Neutrophil Category was not significantly associated in any of the Cox proportional Hazards analysis (Table 8.4) and the confidence intervals for the hazard ratio of the Mid Neutrophil Category always included 1 meaning that the Mid Neutrophil Category was not confidently at greater risk of sustaining a major adverse event than the Low Neutrophil Category.

There was a low probability of breach of the assumptions of the Cox model with Neutrophil count and major adverse event models judged with Cox.zph testing results (0.23 – 0.97, Table 8.4). With a low probability of assumption breach the Cox proportional hazards models are appropriate to use for this data set and generate valid results.

8.3.3. Lymphocyte count associated with major adverse event (death, heart attack or stroke).

8.3.3.1. Kaplan-Meier survival analysis

Kaplan-Meier freedom from major adverse event plot of the cohort is presented in Figure 8.4 by lymphocyte count category. The cohort was divided into categories of lymphocyte count with the quantile formula in S+. This resulted in the Low Lymphocyte Category including baseline lymphocyte cell counts from 0.5 to <1.7 ($x10^9$ cells/L), the Mid Lymphocyte Category including baseline lymphocyte cell counts from 1.7 to <2.33 ($x10^9$ cells/L), and the High Lymphocyte Category including baseline lymphocyte cell counts from 2.33 to 5.6 ($x10^9$ cells/L). Categorisation of the lymphocyte data was done to minimise non-linear effects of the variable on the outcome. There were no missing values for the continuous lymphocyte variable. These categories remain consistent throughout all results chapters.

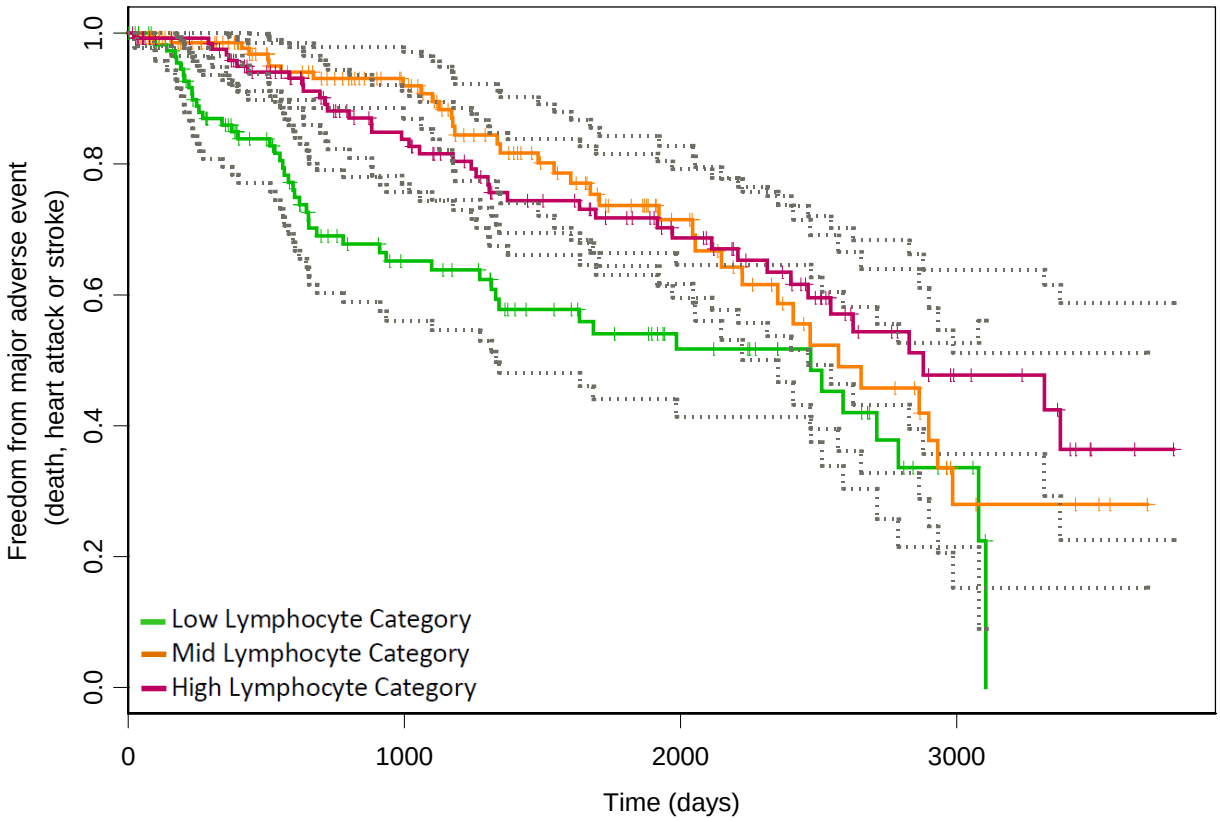

Figure 8.4: Kaplan-Meier non-parametric survival plot - freedom from major adverse event (death, heart attack or stroke) by lymphocyte count category with 95% confidence intervals.

The terminal drop off in the Low Lymphocyte Category in Figure 8.4 is due to the last remaining patient in the category dying at this time. In the other categories the last remaining patients were discharged from follow up without major adverse event.

The Low Lymphocyte Category (green, Figure 8.4) has a distinctly lower freedom from major adverse event plot than the High Lymphocyte Category (red, Figure 8.4). The Low Lymphocyte Category is also mostly lower than the Mid Lymphocyte Category (orange, Figure

8.4) with the exception of an intersection at ~3000 days. The difference between the low and high lymphocyte categories appears visually constant, while the difference between the low and mid lymphocyte categories is greatest between 500 to 2000 days before becoming more closely approximated ~2500 days prior to the intersection discussed above.

The Low Lymphocyte Category has the greatest number of major adverse events over the course of the study (48, 40.7% of the patients in this category) with the lowest mean (1879 ± 128 days = 5.1 years) and median (2471 days = 6.8 years) freedom from major adverse event. The Mid Lymphocyte Category has the lowest number of major adverse events over the course of the study (36, 24.5% of the patients in this category) with mean 2492 ± 132 days (6.8 years) and median 2572 days (7.0 years). The High Lymphocyte Category has the greatest mean (2606 ± 137 days = 7.1 years) and median (2879 days = 7.9 years) freedom from major adverse event experiencing 40 events in a group of 133 patients.

Table 8.5: Kaplan-Meier freedom from major adverse event (death, heart attack or stroke) rate by lymphocyte count category with 95% confidence intervals displayed by days follow up.

Major adverse event		0 days	1000 days	2000 days	3000 days	3500 days
Low Lymphocyte Category	At Risk	118	33	8	5	-
	Events in previous interval	0	33	8	5	-
	Freedom from major adverse event (95% CI)	1.00	0.65 (0.56-0.76)	0.52 (0.41-0.65)	0.34 (0.22-0.53)	-
Mid Lymphocyte Category	At Risk	147	80	32	5	3
	Events in previous interval	0	9	14	13	0
	Freedom from major adverse event (95% CI)	1.00	0.92 (0.87 -0.97)	0.72 (0.62 -0.83)	0.28 (0.15-0.51)	0.28 (0.15-0.51)
High Lymphocyte Category	At Risk	133	77	44	11	2
	Events in previous interval	0	17	12	9	2
	Freedom from major adverse event (95% CI)	1.00	0.84 (0.77-0.91)	0.69 (0.60-0.79)	0.48 (0.36-0.64)	0.36 (0.23-0.59)

CI = Confidence Interval

The Low Lymphocyte Category has the greatest number of major adverse events prior to 1000 days (33) and has the lowest freedom from major adverse events at 1000 and 2000 days (Table 8.5). The Mid Lymphocyte Category has the greatest freedom from major adverse events at 1000 and 2000 days but then the lowest freedom from major adverse events at 3000 days (Table 8.5). This relationship can also be appreciated in Figure 8.4.

Apparent differences in the lymphocyte categories for the outcome of major adverse event with Kaplan-Meir analysis were then analysed with Cox proportional hazards model testing to allow adjustment for traditional and comprehensive risk factors and is seen in Table 8.6.

8.3.3.2. Cox proportional hazards

Table 8.6: Cox proportional hazards analysis - major adverse event and lymphocyte category.

Outcome - major adverse event						
Cell Type		p Value	HR (95% CI)	Other Significant Variables	Cox zph	Global zph
Lymphocyte *a priori*	mid	**<0.01**	**0.52 (0.34-0.80)**	n/a	**<0.01**	**0.03**
	high	**<0.01**	**0.51 (0.33-0.77)**		0.36	
Lymphocyte adjusted TRF[1]	mid	**0.01**	**0.56 (0.36-0.87)**	Age (p=<0.01), IHD (p=0.03)	**0.03**	0.06
	high	**0.01**	**0.58 (0.37-0.90)**		0.40	
Lymphocyte adjusted TRF[1] strata IHD	mid	**0.02**	**0.58 (0.37-0.91)**	Age (p<0.01), Ex-smoker (p=0.05)	**<0.01**	**0.03**
	high	**0.02**	**0.60 (0.38-0.93)**		0.28	
Lymphocyte adjusted TRF[1] strata (IHD & smoking)	mid	**0.03**	**0.61 (0.39-0.96)**	Age (p<0.01)	**<0.01**	**0.05**
	high	**0.04**	**0.62 (0.40-0.97)**		0.11	
Lymphocyte adjusted CRF[2]	mid	**0.04**	**0.62 (0.40-0.98)**	Age (p<0.01), Ex-smoker (p<0.01) IHD (p=0.02), HTN (p=0.03)	**0.02**	0.06
	high	**0.05**	**0.64 (0.41-1.00)**		0.25	
Lymphocyte adjusted CRF[2] strata (HTN, IHD & smoking)	mid	0.11	0.63 (0.42-1.09)	Age (p<0.01)	**0.02**	0.26
	high	0.29	0.75 (0.47-1.25)		0.14	

CI = Confidence Interval n/a = not applicable
IHD = Ischaemic heart disease HTN = Hypertension
TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
strata = statistical stratification for (variable/s in brackets following)
Significant p values at the 0.05 level and corresponding hazard ratio are presented in **bold**

The Mid Lymphocyte Category is significantly inversely associated with the endpoint of major adverse event at the p<0.05 level when tested with the Cox proportional hazards analysis *a priori* (p<0.01, Table 8.6). The hazard ratio of less than one indicates that Mid Lymphocyte category has a protective effect on the outcome of major adverse event with patients in the Mid Lymphocyte Category less likely to sustain a major adverse event than the patients in the Low Lymphocyte Category. When the model was adjusted for traditional risk factors alone (adjusted TRF[1]) Mid Lymphocyte Category was also significantly associated with major adverse events

(p=0.01, Table 8.6) and when this analysis was stratified for the other significant categorical variable ischaemic heart disease, Mid Lymphocyte Category remained significantly inversely associated with major adverse event (p=0.02, Table 8.6). This stratified model revealed another significantly associated categorical variable smoking (p=0.05) so a further stratified model was created. The second stratified model which stratified for ischaemic heart disease and smoking also showed Mid Lymphocyte Category to be significantly inversely associated with major adverse event (p=0.03, Table 8.6). When the model was adjusted for traditional risk factors, disease severity and medication use (adjusted CRF^2) the Mid Lymphocyte category was significantly inversely associated with major adverse event (p=0.04, Table 8.6) however when this adjusted CRF^2 model was stratified for the other significantly associated categorical variables smoking, ischaemic heart disease and hypertension Mid Lymphocyte Category was no longer significantly associated with major adverse event (p=0.11, Table 8.6).

The High Lymphocyte Category is significantly inversely associated with the endpoint of major adverse event at the p=0.05 level when tested with the Cox proportional hazards analysis *a priori* (p<0.01, Table 8.6). The hazard ratio of less than one indicates that High Lymphocyte category has a protective effect on the outcome of major adverse event. When the model was adjusted for traditional risk factors alone (adjusted TRF^1) High Lymphocyte Category was also significantly inversely associated with major adverse events (p=0.02, Table 8.6) and when this analysis was stratified for the other significant categorical variable ischaemic heart disease High Lymphocyte Category remained significantly inversely associated with major adverse event (p=0.02, Table 8.6). This stratified model revealed another significantly associated categorical variable smoking (p=0.05) so a further stratified model was created. The second

stratified model which stratified for ischaemic heart disease and smoking also showed High Lymphocyte Category to be significantly inversely associated with major adverse event (p=0.04, Table 8.6). When the model was adjusted for traditional risk factors, disease severity and medication use (adjusted CRF^2) the High Lymphocyte category was not significantly associated with major adverse event at the 0.05 level and the confidence interval includes 1 (p=0.05, Table 8.6). The association of High Lymphocyte Category with major adverse event was not demonstrated as significant when the adjusted CRF^2 model was stratified for the other significantly associated categorical variables smoking, ischaemic heart disease and hypertension (p=0.29, Table 8.6).

Cox.zph values for the Mid Lymphocyte Category are significant in all models for major adverse event (<0.01 in *a priori* and adjusted TRF^1 models and 0.02 in the adjusted CRF^2 models, Table 8.6) raising the possibility of an assumption violation of these Cox proportional hazards models. This is not unexpected given the Kaplan-Meier results previously that showed the Mid Lymphocyte group experiencing a different path of major adverse events during the course of the study. This is an important finding in that the Mid Lymphocyte group freedom from major adverse event varies over time in a different way to the other groups. This is seen graphically in Figure 8.4 with the Mid Lymphocyte Category initially experiencing a greater freedom from major adverse event rate than the other two lymphocyte categories before descending at a steeper rate than the other two groups, crossing the High Lymphocyte Category at approx. 2000 days and the Low Lymphocyte Category prior to 3000 days so that at the time point of 3000 days the Mid Lymphocyte Category has the lowest freedom from major adverse event. The Cox.zph score alerts us that this is a distinct difference between the groups and is

unlikely due to chance alone, thus the initial protective function of the Mid Lymphocyte Category is greatest compared to the other two groups at 1000 days undergoes a real shift such that the Mid Lymphocyte Category then has the lowest freedom from major adverse event function at 3000 days. The global zph score is also significant in the *a priori* and adjusted TRF[1] models confirming that the overall assumptions of the model are breached by the non-proportionality of the Mid Lymphocyte Category. While the Cox proportional hazards model p values and hazard ratios should be interpreted with this in mind, the most important outcome of this analysis is the dynamic risk of the Mid Lymphocyte Category over time.

8.3.4. Monocyte count associated with major adverse event (death, heart attack or stroke).

8.3.4.1. Kaplan-Meier survival analysis

Kaplan-Meier freedom from major adverse event plot of the cohort is presented in Figure 8.5 by monocyte count category. The categories of monocyte count were derived with the quantile formula in S+. This resulted in the Low Monocyte Category including baseline monocyte cell counts from 0.06 to <0.51 ($x10^9$ cells/L), the Mid Monocyte Category including baseline monocyte cell counts from 0.51 to <0.7 ($x10^9$ cells/L), and the High Monocyte Category including baseline monocyte cell counts from 0.7 to 1.87 ($x10^9$ cells/L). Categorisation of the monocyte data was done to minimise non-linear effects of the variable on the outcome. There were no missing values for the continuous monocyte variable. These categories remain consistent throughout all results chapters.

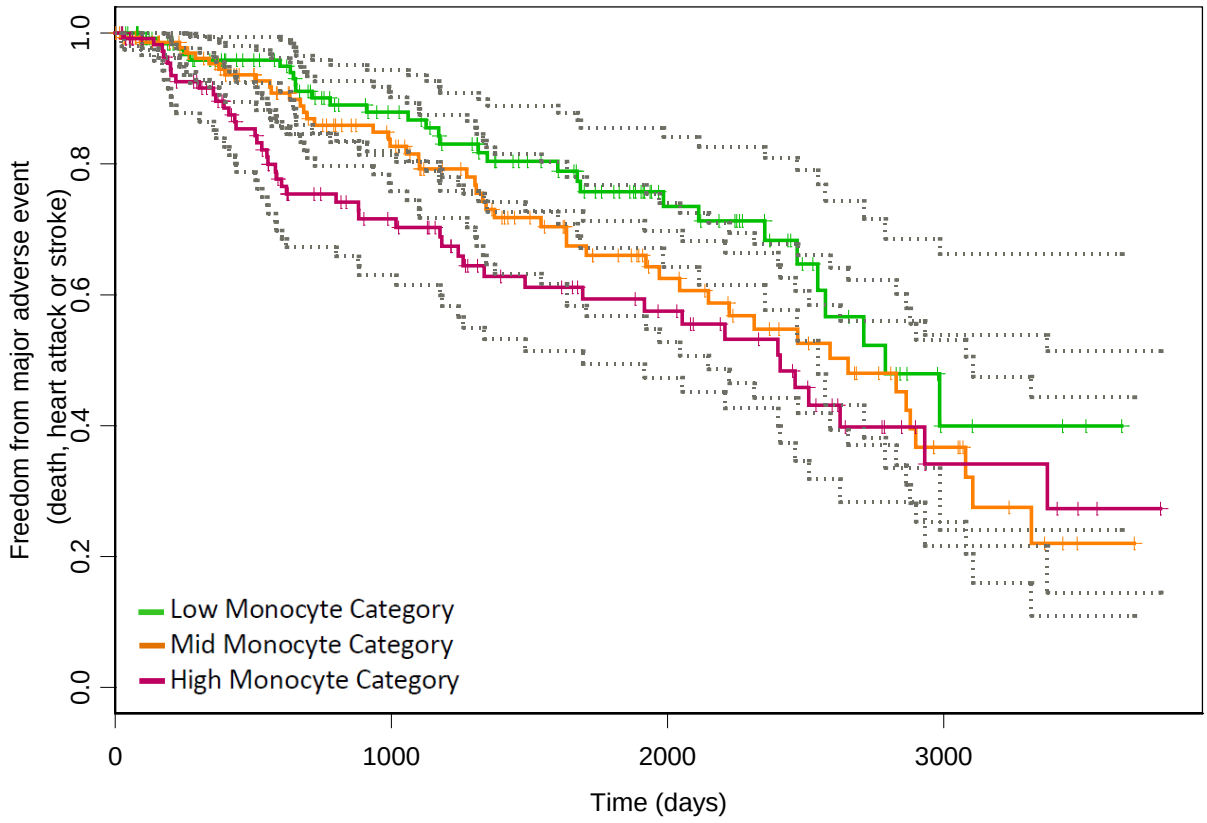

Figure 8.5: Kaplan-Meier non-parametric survival plot - freedom from major adverse event (death, heart attack or stroke) by monocyte count category with 95% confidence intervals.

The High Monocyte Category (red, Figure 8.5) has a visually lower freedom from major adverse event plot which is distinct from the Low Monocyte Category (green, Figure 8.5). The Mid Monocyte Category (orange, Figure 8.5) is mostly between the High and Low Monocyte Categories with the exception of when it crossed and then remains below the High Monocyte Category prior to 3000 days.

The Low Monocyte Category experienced the lowest number of major adverse events over the course of the study (31, 23.1% of the patients in this category) with the greatest mean (2622 ± 139 days = 7.2 years) and median (2544 days = 7.0 years) freedom from major adverse event. The Mid Monocyte Category has the greatest number of major adverse events over the course of the study (48, 33.1% of the patients in this category) with mean of 2341 ± 127 days (6.4 years) and median 2148 (2148 – 3105) days or 5.9 years. The High Monocyte Category had the highest total incidence of major adverse event (37.8% of the patients in this category) and the lowest mean (2182 ± 160 days = 6.0 years) and median (1693 days = 4.6 years) freedom from major adverse event.

Table 8.7: Kaplan-Meier freedom from major adverse event (death, heart attack or stroke) rate by monocyte count category with 95% confidence intervals displayed by days follow up.

Major adverse event		0 days	1000 days	2000 days	3000 days	3500 days
Low Monocyte Category	At Risk	134	75	33	4	2
	Events in previous interval	0	13	10	8	0
	Freedom from major adverse event (95% CI)	1.00	0.88 (0.82-0.94)	0.74 (0.64-0.84)	0.40 (0.24-0.66)	0.40 (0.24-0.66)
Mid Monocyte Category	At Risk	145	76	35	11	1
	Events in previous interval	0	19	15	11	3
	Freedom from major adverse event (95% CI)	1.00	0.83 (0.76-0.90)	0.63 (0.53-0.74)	0.37 (0.25-0.53)	0.22 (0.11-0.44)
High Monocyte Category	At Risk	119	54	30	5	1
	Events in previous interval	0	27	9	8	1
	Freedom from major adverse event (95% CI)	1.00	0.72 (0.60-0.81)	0.58 (0.47-0.70)	0.34 (0.22-0.54)	0.27 (0.15-0.51)

CI = Confidence Interval

The High Monocyte Category has the greatest number of major adverse events prior to 1000 days and has the lowest freedom from major adverse event rate at 1000, 2000 and 3000 days

(Table 8.7). Mid Monocyte Category has the lowest freedom from major adverse event rate at 3500 days. The Low Monocyte Category has the fewest major adverse events prior to 1000 days (13) and has the greatest freedom from major adverse events at all reported time points (Table 8.7). This relationship can also be seen in Figure 8.5.

Apparent differences in the monocyte categories for the outcome of major adverse event with Kaplan-Meir analysis were then analysed with Cox proportional hazards model testing to allow adjustment for traditional and comprehensive risk factors and can be seen in Table 8.8.

8.3.4.2. Cox proportional hazards

Table 8.8: Cox proportional hazards analysis - major adverse event and monocyte category.

Outcome - major adverse event						
Cell Type		p Value	HR (95% CI)	Other Significant Variables	Cox zph	Global zph
Monocyte *a priori*	mid	0.11	1.44 (0.92-2.27)	n/a	0.99	0.09
	high	**<0.01**	**1.85 (1.17-2.92)**		0.08	
Monocyte adjusted TRF[1]	mid	0.21	1.34 (0.85-2.12)	Age (p<0.01), IHD (p=0.02)	0.91	**0.04**
	high	**0.01**	**1.82 (1.13-2.91)**		**0.05**	
Monocyte adjusted TRF[1] strata IHD	mid	0.24	1.32 (0.83-2.09)	Age (p<0.01)	0.97	0.06
	high	**<0.01**	**1.88 (1.18-3.02)**		0.10	
Monocyte adjusted CRF[2]	mid	0.22	1.33 (0.84-2.12)	Age (p<0.01), IHD (p<0.01), Ex-smoker(p=0.02), HTN (p=0.03)	0.74	0.11
	high	**0.02**	**1.81 (1.12-2.92)**		0.19	
Monocyte adjusted CRF[2] strata (IHD, HTN & smoking)	mid	0.16	1.42 (0.87-2.32)	Age (p<0.01)	0.65	0.69
	high	**0.03**	**1.75 (1.07-2.87)**		0.80	

CI = Confidence Interval n/a = not applicable
IHD = Ischaemic heart disease HTN = Hypertension
TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
strata = statistical stratification for (variable/s in brackets following)
Significant p values at the 0.05 level and corresponding hazard ratio are presented in **bold**

The High Monocyte Category is significantly associated with the endpoint of major adverse event at the p<0.05 level when tested with the Cox proportional hazards analysis in all models although the level of significance varies (Table 8.8). In the *a priori* model the High Monocyte Category is significant at the p<0.01 level with a hazard ratio of 1.85 indicating that the High Monocyte Category is 1.85 times more likely than the Low Monocyte Category to sustain a major adverse event (Table 8.8). When the model was adjusted for traditional risk factors alone (adjusted TRF[1]), High Monocyte Category was also significantly associated with major adverse events (p=0.01, Table 8.8) and when this analysis was stratified for the other significant categorical variable ischaemic heart disease, High Monocyte Category remained significantly associated with major adverse event (p<0.01, Table 8.8).

When the Cox model was adjusted for traditional risk factors, disease severity and medication use (adjusted CRF^2) the High Monocyte category was significantly associated with major adverse event (p=0.02, Table 8.8) and when this adjusted CRF^2 model was stratified for the other significantly associated categorical variables smoking, ischaemic heart disease and hypertension High Monocyte Category remained significantly associated with major adverse event (p=0.03, Table 8.8).

The Mid Lymphocyte Category was not significantly different to the Low Lymphocyte Category for the outcome of major adverse event in either the *a priori* or adjusted cox models (Table 8.8).

There was a low probability of breach (0.08–0.99, Table 8.8) of the assumptions of the Cox model with Monocyte count for major adverse event models except the model adjusted for traditional risk factors alone (adjusted TRF^1 - Table 8.8). In the adjusted TRF^1 model the High Monocyte Category had a Cox.zph of 0.05 and the global zph for that model was 0.04 (Table 8.8) raising suspicion that the Cox assumptions of the proportional hazards model are breached when the Low Monocyte Category is compared to the High Monocyte Category and adjusted for traditional risk factors. This is an important finding and tells us that the relationship between these two monocyte categories and the outcome of major adverse event changes over time. While the results of the Cox model should be interpreted with caution in light of the zph breach, this is also a finding that the High Monocyte Category trajectory through the study is not proportional to the Low Monocyte Category for the duration of the study. This is best

understood referring to Figure 8.5 where it can be visibly seen that the High Monocyte Category (red, Figure 8.5) diverges from the Low Monocyte Category (green, Figure 8.5) until approximately 2000 days when the two categories trend closer together. The significant zph score is a measure that this unlikely due to chance alone and likely represents a real change in the relative risk between the groups. One possible explanation is that the pathophysiological benefit of a low monocyte count reduces over time although clarification of this will require further research.

8.3.5. Haemoglobin associated with major adverse event (death, heart attack or stroke).

8.3.5.1. Kaplan-Meier survival analysis

Kaplan-Meier freedom from major adverse event plot of the cohort is presented in Figure 8.6 by haemoglobin category. The cohort was divided into categories of haemoglobin concentration with the quantile formula in S+. This resulted in the Low Haemoglobin Category including baseline haemoglobin concentration from 85 to <131 (g/L), the Mid Haemoglobin Category including baseline haemoglobin concentration from 131 to <146 (g/L), and the High Haemoglobin Category including baseline haemoglobin concentration from 146 to 197 (g/L). Categorisation of the haemoglobin data was done to minimise non-linear effects of the variable on the outcome. There were no missing values for the continuous haemoglobin variable. These categories remain consistent throughout all results chapters.

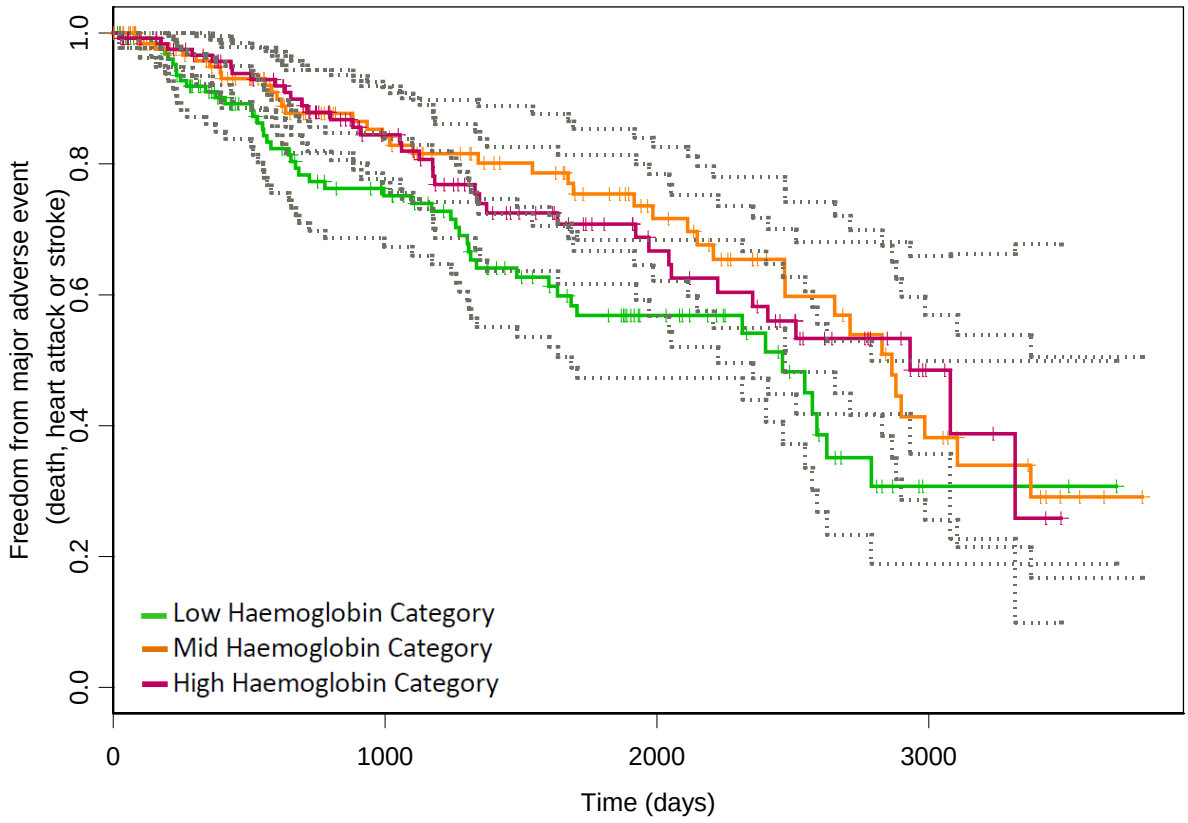

Figure 8.6: Kaplan-Meier non-parametric survival plot - freedom from major adverse event (death, heart attack or stroke) by haemoglobin category with 95% confidence intervals.

Prior to 3000 days the Low Haemoglobin Category (green, Figure 8.6) has a visually lower freedom from major adverse event plot than Mid and High Haemoglobin Categories. The Mid Haemoglobin Category (orange, Figure 8.6) and High Haemoglobin Category (red, Figure 8.6) have visually intertwined freedom from major adverse event plots prior to 1000 days that then separate with the Mid Haemoglobin Category having a higher freedom from major adverse event plot until ~2750 days when they again intersect.

The Low Haemoglobin Category has the greatest number of major adverse events over the course of the study (49, 36.3% of the patients in this category) with the shortest mean (2174 ± 145 days = 6.0 years) and median (2462 days (1685 – 2789) = 6.7 years) freedom from major adverse event. The High Haemoglobin Category has the least number of major adverse events over the course of the study (37, 29.1% of the patients in this category) and the greatest median freedom from major adverse event (2931= 8.0 years) and a mean of 2412 ± 129 days (6.6 years). The Mid Haemoglobin Category has the greatest mean freedom from major adverse event (2563 days = 7.0 years) with a total of 38 major adverse events in a group of 136 patients and a median freedom from major adverse event of 2864 days (7.8 years)

Table 8.9: Kaplan-Meier freedom from major adverse event (death, heart attack or stroke) rate by haemoglobin category with 95% confidence intervals displayed by days follow up.

Major adverse event		0 days	1000 days	2000 days	3000 days	3500 days
Low Haemoglobin Category	At Risk	135	67	29	2	2
	Events in previous interval	0	27	14	8	0
	Freedom from major adverse event (95% CI)	1.00	0.75 (0.67-0.84)	0.57 (0.47-0.68)	0.31 (0.19-0.50)	0.31 (0.19-0.50)
Mid Haemoglobin Category	At Risk	136	69	37	12	3
	Events in previous interval	0	16	8	12	2
	Freedom from major adverse event (95% CI)	1.00	0.84 (0.77-0.92)	0.72 (0.62-0.83)	0.38 (0.26-0.57)	0.29 (0.17-0.51)
High Haemoglobin Category	At Risk	127	69	32	6	-
	Events in previous interval	0	16	12	7	-
	Freedom from major adverse event (95% CI)	1.00	0.84 (0.78-0.92)	0.67 (0.57-0.78)	0.49 (0.36-0.66)	-

CI = Confidence Interval Hb= Haemoglobin

The Low Haemoglobin Category has the greatest number of major adverse events prior to 1000 days (27) and has the lowest freedom from major adverse event rate at all 1000, 2000, 3000

but not 3500 day time points (Table 8.9). The Mid Haemoglobin Category has the greatest freedom from major adverse event rate at 1000 and 2000 days but at 3000 days it is the High Haemoglobin Category that has the highest freedom from major adverse event (Table 8.9). The Mid Haemoglobin Category then achieves the lowest freedom from major adverse event rate by 3500 days (Table 8.9). This relationship can also be seen in Figure 8.6.

Apparent differences in the haemoglobin categories for the outcome of major adverse event with Kaplan-Meir analysis were then analysed with Cox proportional hazards model testing to allow adjustment for traditional and comprehensive risk factors and can be seen in Table 8.10.

8.3.5.2. Cox proportional hazards

Table 8.10: Cox proportional hazards analysis - major adverse event and haemoglobin category.

Outcome - major adverse event						
Haemoglobin Level		p Value	HR (95% CI)	Other Significant Variables	Cox zph	Global zph
Haemoglobin *a priori*	mid	**0.04**	**0.63 (0.41-0.97)**	n/a	0.33	0.63
	high	0.06	0.66 (0.43-1.02)		0.64	
Haemoglobin adjusted TRF[1]	mid	0.37	0.81 (0.52-1.28)	Age (p<0.01), IHD (p=0.03)	0.37	0.13
	high	0.82	0.94 (0.58-1.54)		0.54	
Haemoglobin adjusted TRF[1] strata IHD	mid	0.38	0.82 (0.52-1.29)	Age (<0.01)	0.34	0.11
	high	0.83	0.95 (0.58-1.55)		0.59	
Haemoglobin adjusted CRF[2]	mid	0.86	0.92 (0.59-1.57)	Age (p<0.01), IHD (p=0.01), Ex-smoker (p=0.02), HTN (p=0.02),	0.97	0.14
	high	0.89	1.01 (0.62-1.74)		0.46	
Haemoglobin adjusted CRF[2] strata (Smoking, IHD &HTN)	mid	0.91	0.93 (0.61-1.74)	Age (p<0.01)	0.33	0.44
	high	0.76	1.03 (0.66-1.88)		0.90	

CI = Confidence Interval n/a = not applicable
IHD = Ischaemic heart disease HTN = Hypertension
TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
strata = statistical stratification for (variable/s in brackets following)
Significant p values at the 0.05 level and corresponding hazard ratio are presented in **bold**

The Mid Haemoglobin Category is significantly associated with the endpoint of major adverse event at the p<0.05 level when tested with the Cox proportional hazards analysis *a priori* (p=0.04, Table 8.10). The hazard ratio of less than one (p=0.63, Table 8.10) indicates that Mid Haemoglobin Category has a protective effect on the outcome of major adverse event compared to the Low Haemoglobin Category. When the model was adjusted for traditional risk factors alone (adjusted TRF[1]) and adjusted for comprehensive risk factors: traditional risk factors, disease severity and medication use (adjusted CRF[2]), neither Mid or High Haemoglobin category was significantly associated with major adverse event (Table 8.10).

There was a low probability of breach of the assumptions of the Cox model with Haemoglobin count and major adverse event models assessed with Cox.zph testing results (0.11 – 0.90, Table 8.10). With a low probability of assumption breach the Cox proportional hazards models are appropriate to use for this data set and generate valid results.

8.3.6. Neutrophil-Lymphocyte Ratio associated with major adverse event (death, heart attack or stroke).

8.3.6.1. Kaplan-Meier survival analysis

Kaplan-Meier freedom from major adverse event plot of the cohort is presented in Figure 8.7 by neutrophil-lymphocyte ratio (NLR) category. The cohort was divided into categories of NLR with the quantile formula in S+. This resulted in the Low NLR Category including baseline NLR from 0.73 to <1.89, the Mid NLR Category including baseline NLR from 1.89 to <2.64, and the High NLR Category including baseline NLR from 2.64 to 20.87. Categorisation of the NLR data was done to minimise non-linear effects of the variable on the outcome. It is worth noting that there were no missing values for the continuous NLR variable. These categories remain consistent throughout all results chapters.

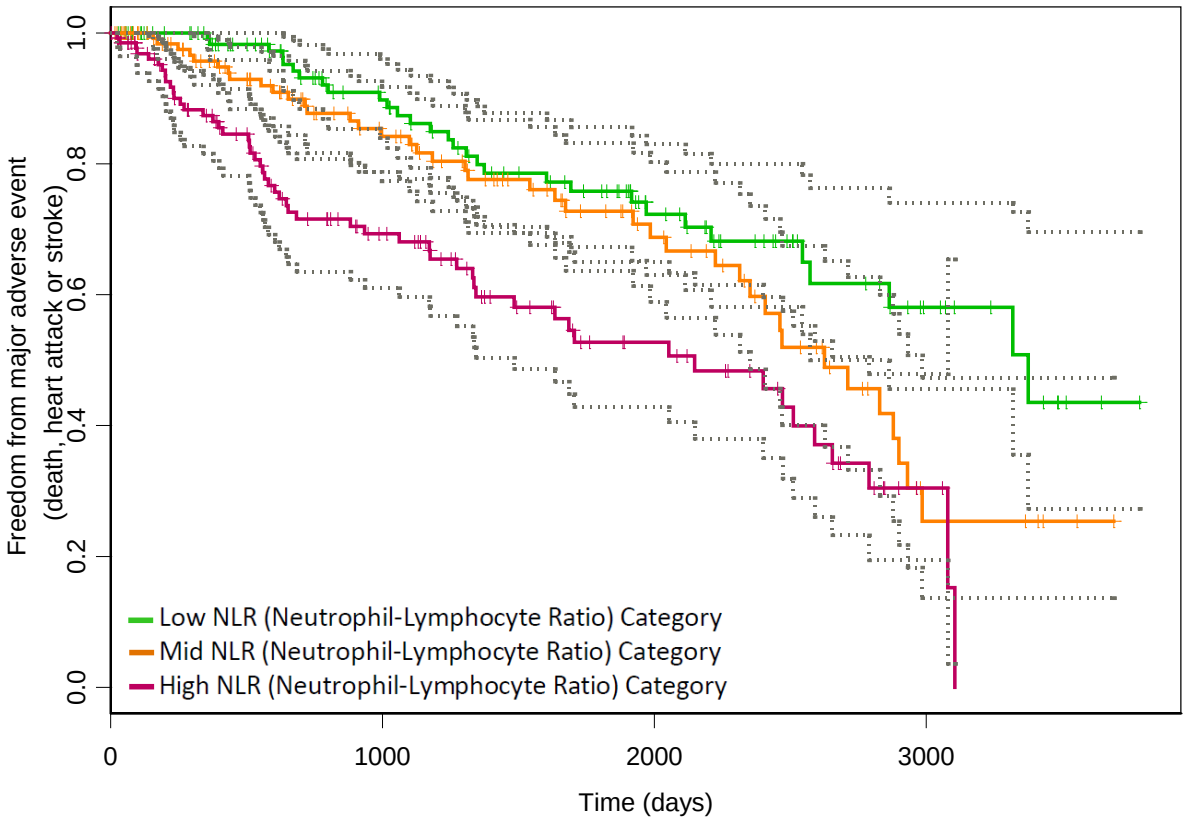

Figure 8.7: Kaplan-Meier non-parametric survival plot - freedom from major adverse event (death, heart attack or stroke) by neutrophil-lymphocyte ratio category with 95% confidence intervals.

All three NLR categories have visually distinct freedom from major adverse event plots (Figure 8.7) with the exception of an intersection of the High NLR Category (red, Figure 8.7) and the Mid NLR Category (orange, Figure 8.7) just prior to 3000 days. The terminal drop off in the High NLR Category (red, Figure 8.7) is due to the last remaining patient in the High NLR Category sustaining a major adverse event at this time. In the Mid and Low NLR Categories the last remaining patients were discharged from follow up without sustaining a major adverse event.

The High NLR Category has the greatest number of major adverse events over the course of the study (54, 40.6% of the patients in this category) with the shortest mean (1859 ± 119 days = 5.1 years) and median (2148 days (1484 – 2789) = 5.9 years) freedom from major adverse event. The Low NLR Category has the least number of major adverse events over the course of the study (30, 22.6% of the patients in this category) and the greatest median freedom from major adverse event (2572 days = 7.0 years) and the greatest mean freedom from major adverse event (2811 ± 139 days = 7.7 years). The Mid NLR Category sustained 40 events from a population of 132 patients with a mean of 2411 ± 132 days (6.6 years) and a median of 2352 (2352 – 2985) days (6.4 years).

Table 8.11: Kaplan-Meier freedom from major adverse event (death, heart attack or stroke) rate by neutrophil-lymphocyte ratio category with 95% confidence intervals displayed by days follow up.

Major adverse event		0 days	1000 days	2000 days	3000 days	3500 days
Low NLR Category	At Risk	133	77	39	12	3
	Events in previous interval	0	10	13	5	2
	Freedom from major adverse event (95% CI)	1.00	0.89 (0.84-0.96)	0.72 (0.63-0.83)	0.58 (0.46-0.74)	0.44 (0.27-0.70)
Mid NLR Category	At Risk	132	70	34	5	2
	Events in previous interval	0	16	10	14	0
	Freedom from major adverse event (95% CI)	1.00	0.84 (0.77-0.92)	0.69 (0.59-0.80)	0.25 (0.14-0.47)	0.25 (0.14-0.47)
High NLR Category	At Risk	133	58	25	3	-
	Events in previous interval	0	33	11	8	-
	Freedom from major adverse event (95% CI)	1.00	0.69 (0.61-0.79)	0.52 (0.43-0.65)	0.30 (0.19-0.48)	-

CI = Confidence Interval NLR = Neutrophil-lymphocyte ratio

The High NLR Category has the greatest number of major events in the first 1000 days (33) and continues with the lowest freedom from major adverse event at 1000 and 2000 days (Table 8.11). The lowest freedom from major adverse event at 3000 is the Mid NLR Category (Table 8.11). The Low NLR Category has the greatest freedom from major adverse event at each reported time point (Figure 8.7 and Table 8.11).

Apparent differences in the NLR categories for the outcome of major adverse event with Kaplan-Meir analysis were then analysed with Cox proportional hazards model testing to allow adjustment for traditional and comprehensive risk factors and can be seen in Table 8.12.

8.3.6.2. Cox proportional hazards

Table 8.12: Cox proportional hazards analysis - major adverse event and neutrophil/lymphocyte ratio category.

Outcome - major adverse event						
Cell Ratio		p Value	HR(95% CI)	Other Significant Variables	Cox zph	Global zph
NLR *a priori*	mid	0.07	1.54 (0.96-2.48)	n/a	0.58	0.34
	high	**<0.01**	**2.70 (1.72-4.25)**		0.45	
NLR adjusted TRF[1]	mid	0.28	1.31 (0.17-1.49)	Age (p<0.01)	0.88	0.11
	high	**<0.01**	**2.10 (0.73-4.32)**		0.34	
NLR adjusted CRF[2]	mid	0.32	1.29 (0.78-2.14)	Age (p<0.01), Ex-smoker (p<0.01), IHD (p=0.01), HTN (p=0.02), Smoker (p=0.03)	0.92	0.12
	high	**0.02**	**1.80 (1.11-2.91)**		0.27	
NLR adjusted CRF[2] strata (smoking, HTN, IHD)	mid	0.48	1.21 (0.72-2.04)	Age (p<0.01)	0.28	0.25
	high	0.09	1.56 (0.93-2.61)		0.36	

CI = Confidence Interval NLR = Neutrophil-lymphocyte ratio n/a = not applicable
IHD = Ischaemic heart disease HTN = Hypertension
TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
strata = statistical stratification for (variable/s in brackets following)
Significant p values at the 0.05 level and corresponding hazard ratio are presented in **bold**

The High NLR Category is significantly associated with the endpoint of major adverse event at the p<0.05 level when tested with the Cox proportional hazards analysis *a priori* (p<0.01, Table 8.12). The hazard ratio of 2.70 indicates that without taking into account other risk factors patients in the High NLR Category are 2.7 times more likely to sustain a major adverse event than patients in the Low NLR Category. When the model was adjusted for traditional risk factors alone (adjusted TRF[1]) High NLR Category was also significantly associated with major adverse events (p<0.01, Table 8.12) although the hazard ratio reduced to 2.10 (Table 8.12). When the model was adjusted for comprehensive risk factors: traditional risk factors, disease severity and medication use (adjusted CRF[2]), the High NLR category was again

significantly associated with major adverse event (p=0.02, Table 8.12) and the hazard ratio remained high (HR=1.80, Table 8.12).

The association of High NLR Category with major adverse event was trended toward association but was not significantly associated at the $p<0.05$ level with major adverse event when the adjusted CRF^2 model was stratified for the other significantly associated categorical variables smoking, ischaemic heart disease and hypertension (p=0.09, Table 8.12).

The Mid NLR Category was not significantly associated with major adverse event in any of the Cox models either *a priori* or when adjusted for risk factors.

There was a low probability of breach of the assumptions of the Cox model with Neutrophil/Lymphocyte Ratio Category and major adverse event models as assessed by the Cox.zph testing results (0.11 – 0.92, Table 8.12). With a low probability of assumption breach the Cox proportional hazards models are appropriate to use for this data set and generate valid results.

8.3.7. Summary of circulating cell types Cox proportional hazards analysis for major adverse event

All previously displayed single circulating cell type Cox proportional hazard models for the outcome of major adverse event are summarised in Table 8.13.

Table 8.13: Single cell type Cox proportional hazards analysis summary - major adverse event.

Major Adverse Event						
Cell Type		*a priori*	Adjusted TRF	Adjusted TRF strata	Adjusted CRF	Adjusted CRF strata
TWCC Category	mid	*	NS	NS	**	*
	high	*	*	*	*	**
Neutrophil Category	mid	NS	NS	NS	NS	NS
	high	**	**	**	**	*
Lymphocyte Category	mid	**	**	*	*	NS
	high	**	**	*	*	NS
Monocyte Category	mid	NS	NS	NS	NS	NS
	high	**	**	**	*	*
Haemoglobin Category	mid	*	NS	NS	NS	NS
	high	#	NS	NS	NS	NS
NLR Category	mid	#	NS	n/a	NS	NS
	high	**	**	n/a	*	#

NS = Not significant
# = p value approaching significance = 0.1 to 0.05
* = p value = 0.01 to 0.05
** = p value <0.01
n/a = not applicable
TWCC = Total white cell count
NLR = Neutrophil-lymphocyte ratio
TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
strata = statistical stratification for other significant categorical variables

The level of significance for the circulating cell type in the various Cox proportional hazards models (Table 8.13) depends in all cases on the variables adjusted for and the statistical method of dealing with statistically significant co-variables. The cell types most consistently significantly associated across all models are the High TWCC category, the High Neutrophil Category and the High Monocyte Category, which are significantly associated with the

outcome of major adverse event in all models. The High Neutrophil-Lymphocyte Ratio (NLR) category is significantly associated with major adverse event in the *a priori* model and when adjusting for traditional risk factors but only trending to significance when the models are adjusted for comprehensive risk factors (Table 8.13). The Mid Neutrophil Category and the Mid Monocyte Category were not significantly associated with major adverse event in any model.

Table 8.14: Combined cell types Cox proportional hazards analysis – major adverse event and all cell types by category.

Major adverse event					
Cell Type		p Value	HR (95% CI)	Cox.zph	Global zph
Neutrophil Category	mid	0.24	1.37 (0.81-2.30)	0.83	0.08
	high	**0.03 ***	**1.77 (1.05-2.98)**	0.83	
Lymphocyte Category	mid	**<0.01 ****	**0.50 (0.32-0.79)**	**<0.01**	
	high	**<0.01 ****	**0.40 (0.25-0.64)**	0.10	
Monocyte Category	mid	0.18	1.40 (0.86-2.30)	0.69	
	high	**<0.01 ****	**2.04 (1.19-3.48)**	0.04	
Haemoglobin Category	mid	0.06 #	0.66 (0.42-1.00)	0.51	
	high	**0.05 ***	**0.64 (0.41-0.99)**	0.95	

= p value = 0.1 to 0.05
* = p value = 0.01 to 0.05 (**bold** type)
** = p value <0.01(**bold** type)
HR = Hazard Ratio
NS = Not significant

A combined circulating cell types Cox proportional hazards model was used to assess all cell subtypes and the outcome of major adverse event tested *a priori* and the results are displayed in Table 8.14. Multiple cell categories were significantly associated with the outcome of major adverse event and are represented in Table 8.14 in **bold** type. The High Neutrophil Category was significantly associated with major adverse event with (p=0.03, Table 8.14) with the hazard ratio of 1.77 indicating that patients in the High Neutrophil Category are 1.77 times more likely to sustain a major adverse event than patients in the Low Neutrophil Category.

Both Mid and High Lymphocyte Categories were significantly negatively associated with major adverse event ($p<0.01$, Table 8.14). Both Mid and High Lymphocyte Categories have a hazard ratio range less than one that indicating that these categories of lymphocyte count have reduced risk of sustaining a major adverse event when compared to the Low Lymphocyte Category (Table 8.14).

High Monocyte Category was also significantly associated with major adverse event in this model ($p<0.01$, Table 8.14). In the case of High Monocyte Category the hazards ratio of 2.04 indicates that patients in the High Monocyte Category were more than twice as likely to have a major adverse event as those patients in the Low Monocyte Category.

High Haemoglobin Category was also significantly associated with major adverse event in this model ($p=0.05$, Table 8.14). High Haemoglobin Category has a hazards ratio range less than one indicating a protective association with major adverse event. It is also worth noting that the Mid Haemoglobin Category was only just outside the chosen range of significance and the 95% confidence interval for the hazard ratio only just includes one (Table 8.14).

This model was not adjusted for confounding variables as with previous models of only one cell type due to the eight degrees of freedom already required for this model. Population and event incidence do not allow for meaningful adjustment with either traditional risk factors (TRF) or comprehensive risk factors (CRF) due to the number of degrees of freedom these models would require being beyond the power of this study.

The probability of breach of the assumptions of the Cox model for all circulating cell types and major adverse event models was raised with both Mid Lymphocyte Category ($p<0.01$, Table 8.14) and High Monocyte Category ($p=0.04$, Table 8.14) significant at the $p<0.05$ level. As discussed previously this is most likely due to a change in the relationship of the Mid Lymphocyte Category and High Monocyte Category with the endpoint of major adverse event over time. This raises suspicion that the Cox proportional hazards models may not generate valid results for this data set and caution should be used when interpreting the results. It was determined that further analysis of cell counts, risk factors and the outcome of major adverse event was warranted by the likelihood of *a priori* relationships of circulating cell types and the outcome of major adverse event. Transformation of the data and analysis with other semiparametric testing was considered, however after reviewing the nature of complex interactions of the cell types and risk factors it was determined that analysis that not only allows for term interaction, but is able to statistically account for that interaction was more appropriate. This was done using multi-model averaging and is presented below.

8.3.8. Multi-model analysis for the outcome of major adverse event

For the outcome of major adverse event the traditional risk factors of entry age, gender, diabetes mellitus, hypertension, ischaemic heart disease, transient ischaemic attack and stoke as well as disease severity at presentation and the medications of aspirin and statin were included in multi-model analysis with cell counts Neutrophil Category, Lymphocyte Category, Monocyte Category, Haemoglobin Category and calculated Neutrophil/Lymphocyte Ratio Category. These variables were selected for the multi-model analysis due to their significant *a priori* associations with the outcome of major adverse event demonstrated with Cox proportional hazards analysis presented earlier in this chapter (Table 8.13) establishing the relationship of these variables with the outcome of major adverse event.

The statistical package R[354] was used to run all possible models using the explanatory variables above for the outcome of major adverse event with the package MuMIn[355] (with K=15 parameters described above). For the outcome of major adverse event the variable of gender had a very low frequency in one category and required stratification to exclude unintended influence on the analysis. The variable of smoking was also found to be better represented for the purpose of multi-model analysis when the three categories of never smoking, ex-smoking and current smoking were reduced to only two categories of never smoker and smoker (previous or current).The models were then reduced to just the models that were not significantly different to the "best" single model using the delta Δ_i <2 function (Table 8.15). The results of this analysis are presented in Table 8.15 with all the information required to interpret the analysis.[368] Background information and equations are found in Chapter 4 summarised in Table 4.3.

Table 8.15: Multi-model analysis: top model set for major adverse event (Δ_i <2)

Component models:	df	log($\mathcal{L}$)	AIC_c	Δ_i	w_i
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category/strata(gender)	10	-507.18	1034.93	0.00	0.10
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / Neutrophil Category / strata(gender)	12	-505.17	1035.15	0.22	0.09
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category / strata(gender)	10	-507.51	1035.59	0.66	0.07
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / strata(gender) / TIA	11	-506.47	1035.63	0.70	0.07
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / Neutrophil Category / Smoking / strata(gender)	13	-504.50	1035.94	1.01	0.06
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category / Smoking / strata(gender)	11	-506.65	1035.98	1.05	0.06
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / Smoking/ strata(gender)	11	-506.74	1036.16	1.23	0.06
Age / HTN / IHD / Disease Severity / Neutrophil Category / strata(gender)	8	-509.93	1036.24	1.31	0.05
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / Statin / strata(gender) / DM	11	-506.86	1036.40	1.47	0.05
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / strata(gender)	11	-506.90	1036.48	1.55	0.05
Age / HTN / IHD / Disease Severity / Neutrophil Category / Smoking / strata(gender)	9	-509.06	1036.58	1.65	0.05
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / Neutrophil Category / strata(gender) / TIA	13	-504.86	1036.66	1.73	0.04
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / Smoking / strata(gender) / TIA	12	-505.94	1036.68	1.75	0.04
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / Neutrophil Category / Statin / strata(gender)	13	-504.91	1036.76	1.83	0.04
Age / HTN / Disease Severity / Lymphocyte Category / Neutrophil Category / strata(gender)	9	-509.17	1036.80	1.87	0.04
Age / HTN / Disease Severity / Lymphocyte Category / Monocyte Category / Neutrophil Category / strata(gender)	11	-507.06	1036.81	1.88	0.04
Age / HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / Neutrophil Category / strata(gender) / DM	13	-504.94	1036.84	1.91	0.04
Age / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / strata(gender)	9	-509.22	1036.91	1.98	0.04

AIC_c = Akaike information criterion corrected for sample size (Equation 10)
Δ_i = AICi-AICmin (Equation 11)
log($\mathcal{L}$) = Log likelihood of individual model ($\mathcal{L}$ from Equation 12)
w_i = Akaike weight (Equation 13)
IHD = Ischaemic Heart Disease
Disease Severity = Disease Severity at presentation
Smoking = Smoking Category (Never smoker, Ex-smoker, Current smoker)
The two top models are highlighted.
df = Degrees of Freedom
DM = Diabetes Mellitus
HTN = Hypertension

The models not significantly worse than the "best" model (Δ_i <2) were selected from the whole set of models for the data set with the outcome major adverse event, these 18 top models (Δ_i <2) are presented in Table 8.15. The simplest model had only 8 degrees of freedom while four models had 13 degrees of freedom. The log($\mathcal{L}$) values range from -504.50 to -509.93 demonstrating comparable fit of the selected models. The individual AIC_c are not interpretable as they contain arbitrary constrains and are greatly affected by sample size[367] but once transformed to Δ_i with the best model given a Δ value of 0 and three other models having a Δ_i <1 the information loss in using those models over the "best" model is quantified.

The "best" model contained the variables age, hypertension, ischaemic heart disease, disease severity, lymphocyte category and monocyte category with gender stratified (highlighted in Table 8.15). The "best" model has been estimated to be the best but the w_i results show that no one model is supported as being clearly superior over the other considered models with the "best" model having a probability of only 10% and the second model 9% and all other models from the top model set (with Δ_i <2) $\geq$ 4%. The w_i / w_j evidence support ratio demonstrates the empirical support for the "best" model is 2.5 times that of the 7 models with w_i = 0.04 and twice that of the 4 models with w_i = 0.05.

The second best model for the outcome of major adverse event contained the Neutrophil Category in addition to the same variables contained within the best model and results in a Δ_i of 0.22 which may be interpreted as having minimal information loss despite the requirement of two additional degrees of freedom. The w_i / w_j evidence support ratio confirms the empirical support for the "best" model is only 1.1 times that of the second best model.

Multi-model averaging is recommended to account for model selection uncertainty when the w_i of the best model is <0.90.[132,372] Multi-model averaging was undertaken which has the benefit of being able to include important information not included in the "best" model and account for model selection uncertainty in calculating robust parameter estimates or predictions.[372] Multi-model averaging was then applied to make inference from all of the models with Δ_i <2 with adjustment for their supportive value and model probability. The variables from all the models in Table 8.15 were thus ranked according to the number of models (from the top model set with Δ_i <2) that each variable appeared in and factored for the complexity of each of those models using the Akaike weight for the model (w_i) to calculate the importance for each variable for the outcome of major adverse event. The results of ranking variables using this process are presented in Table 8.16 and is particularly important in this analysis due to the similar levels of support for the top models (Table 8.15). The subsequent averaged model is then analysed to assess the coefficients and significance of contributing variables to the averaged model (Table 8.17).

Table 8.16: Relative variable importance from multi-model analysis for major adverse event

Variable	Importance	No. of models
Age	1.00	18
Disease Severity	1.00	18
Strata (Gender)	1.00	18
Hypertension	0.96	17
Ischaemic Heart Disease	0.92	16
Lymphocyte Category	0.90	16
Monocyte Category	0.73	13
Neutrophil Category	0.59	11
Smoking	0.27	5
Transient Ischaemic Attack	0.16	3
Statin	0.09	2
Diabetes Mellitus	0.09	2

Table 8.16 shows that eight variables (Entry Age, Disease severity at presentation, Stratified Gender, Hypertension, Ischaemic Heart Disease, Lymphocyte Category, Monocyte Category and Neutrophil Category) were present in more than half of the models in the top model set with Δ_i <2. These variables are the best predictors for the outcome of major adverse event in the population. The "best" model contained seven of these eight variables.

The lower relative importance of smoking and that it only appeared in five models in the top model set implies that it is less likely to be a good predictor of major adverse event than the best predictors referred to earlier but may still have significant influence in the models in which it was included. Of the models that included smoking two of the five did not include Neutrophil Category. Transient ischaemic attack, statin use and diabetes mellitus were present in the fewest models and are less likely to be strong predictors of major adverse event compared to the best predictors. Two of the three models that contained Transient Ischaemic Attack did not contain Neutrophil Category. One model that did not contain Neutrophil category contained both statin use and diabetes.

Neutrophil-Lymphocyte Ratio has the advantage of less degrees of freedom than including both the Neutrophil Category and Lymphocyte Category however this variable was not in any of the models from the top model set with Δ_i <2, although both Neutrophil Category and Lymphocyte Category appear together in 10 of the 18 models.

The model averaged coefficients were generated using the natural average method[132,372] and are displayed in Table 8.17. The coefficients indicate the direction of effect for each variable with size of standard error, i.e. a negative coefficient indicates a negative effect on the outcome of major adverse event, that the event is less likely to occur.

Table 8.17: Multi-model inference averaged coefficients for major adverse event

Variable	Standardised Coefficient	SE of coefficient	z value	Pr(>|z|)
Rest pain	0.71	0.59	1.19	0.24
High Monocyte Category	0.56	0.43	1.29	0.20
High Lymphocyte Category	-0.53	0.30	1.78	0.08
Hypertension	-0.53	0.28	1.90	0.06
Mid Lymphocyte Category	-0.49	0.28	1.76	0.07
Tissue Loss	0.41	0.58	0.70	0.49
High Neutrophil Category	0.39	0.39	0.98	0.32
Intermittent claudication	-0.39	0.54	0.74	0.46
Ischaemic Heart Disease	0.38	0.23	1.66	0.09
Mid Monocyte Category	0.23	0.27	0.87	0.39
Mid Neutrophil Category	0.18	0.26	0.70	0.49
Age	0.06	0.01	5.32	<0.001
Transient Ischaemic Attack	-0.05	0.17	0.30	0.76
Smoking	0.04	0.09	0.42	0.67
Statin	-0.01	0.07	0.19	0.85
Diabetes Mellitus	-0.01	0.07	0.17	0.86

SE = Standard error

Table 8.17 presents the multi-model inference results for the averaged model which combined the top model set of Δ_i <2 candidate models weighted by their support. The standardised coefficient and standard error were used to generate a z value and the probability of each z

value (Pr(>|z|)) are included in Table 8.17. Caution needs to be taken in interpreting these results from a null hypothesis testing paradigm that traditionally uses the probabilities of z statistics to accept or reject the null hypothesis.[132,368] These results are included here to demonstrate the relative effect of coefficient and standard error on the individual variables in the averaged model and not in the binary significant/not significant way.[368] The z value is one way of quantifying the effect of a variable in the averaged model that accounts for the magnitude of the adjusted coefficient and allows comparison of coefficients with different standard errors. Importantly, variables with a non-significant Pr value are not discarded, because the overall averaged model is better with them than without them but in the averaged model they have weaker effects on the outcome of major adverse event than variables with smaller Pr values. The variable with the smallest Pr value was Age which was in all of the top model set, and the effect of age in the "best model" and subsequent averaged model is not substitutable by any of the other variables. The Pr values are very useful in the interpretation of the averaged model for variables that are likely to be correlated and hence partly substitutable in the averaged model. The low Pr values of High Lymphocyte Category, Mid Lymphocyte Category, Hypertension and Ischaemic Heart Disease along with the presence of these variables in most or all of the top model set may be interpreted as these variables being key or non-substitutable in the averaged model for major adverse event. High Neutrophil Category demonstrated a comparatively larger Pr value of 0.49 (Table 8.17) and was only in 11 of the 18 models in the top model set (Table 8.16). Of the seven models that did not contain Neutrophil Category in Table 8.15: two contain Smoking, two contain TIA and one contains Diabetes Mellitus, none of which were represented in the "best model". Smoking and Neutrophil Category are expected from previously published literature to be correlated,[383]

therefore when they included in the same model it is expected to see that both will have their level of significance reduced or eliminated compared to if only one of these variables was included.

The averaged coefficients presented in Table 8.17 will always be weaker than the coefficients in the "best" model but the process is designed to be generous with inclusion over exclusion and will tend to underestimate the relative strength of variable effect compared with the "best" model.

From the categorical variables, Rest Pain has the largest coefficient (0.71, Table 8.17), which is best described as the magnitude of effect. Rest Pain therefore exhibits the largest effect on the model for the outcome of major adverse event. Rest pain does have a standard error of 0.59 which is why the z value is 1.19 (Pr=0.24, Table 8.17). This z value and corresponding p value do not mean that this is not an important (or non-significant) result. It is instead a method of quantifying the largest variable effect in the averaged model also having a large standard error.

High Monocyte Category demonstrated the largest effect of the circulating cell types in the averaged model which was greater than all traditional risk factors included. Again the standard error of High Monocyte Category approaches the coefficient resulting in a z value of 1.29 (Table 8.17). High Lymphocyte Category was shown to have the same effect size as the traditional risk factor of hypertension with both coefficients negative indicating a protective effect for the outcome of major adverse event. With both High Lymphocyte Category and Hypertension having the same coefficient the effect of a smaller standard error for the variable

of hypertension can be seen to result in a larger z value of 1.90 (Pr=0.06, Table 8.17). Mid Lymphocyte Category also displayed a negative coefficient larger than the rest of the traditional risk factors (i.e. other than hypertension).

High and Mid Neutrophil category and Mid Monocyte category all demonstrated larger coefficients than the other traditional risk factors of transient ischaemic attack, smoking, statin and diabetes mellitus. Hypertension, High and Mid Lymphocyte Category and Intermittent claudication all have a negative coefficient or are protective for the outcome of major adverse event.

In the averaged model Tissue Loss has a coefficient of 0.41 but the standard error of this value is greater resulting in a z value <1. High Neutrophil category carried the same variable weighting as the disease severity category of intermittent claudication, although High Neutrophil Category was positively associated with the outcome of major adverse event and Intermittent claudication was protective for this outcome.

Ischaemic Heart Disease is the ninth strongest variable for the outcome of major adverse event (coefficient 0.38) but with relatively small standard error of 0.23 (Table 8.17).

Age is the only continuous variable in this multi-model analysis and although the coefficient is seeming more modest than other variables (0.06, Table 8.17) it has the smallest standard error and as such generated the largest z value. The coefficient for age applies to the value for that continuous variable and it has a strong effect on the outcome in this case major adverse

event. For example in an 80 year old patient the age term would be a function of the coefficient (0.06, Table 8.17) and the value 80.

The four weakest standardised coefficients also belong to the variables that feature in the least number of best fit models (Table 8.16) and interestingly these are all traditional risk factors that currently constitute models for major adverse event in patients with peripheral arterial occlusive disease. It is possible that transient ischaemic attack, smoking, diabetes mellitus and statin use interact with one or more of the other variables in the model (e.g. smoking and/or neutrophil count[383]) and the better predictor may suffice but in the interests of caution these variables have been included in the average model because it is not possible to tell from this data set alone which of these variables can be safely excluded. Care should be exercised in interpreting the direction of association of these weakly correlated variables as assigning the incorrect sign to a weak parameter is a possibility in any inference analysis.[384,385]

8.4. Discussion for MAE

The aim of this study was to combine the assessment of all the circulating cell types for the composite endpoint of major adverse event, and to generate a predictive model for the outcome of major adverse event that combines the circulating cell count values with established risk factors and clinical disease severity. This is the first study to apply the Information-Theoretical paradigm and in particular multi-model inference to a population of patients with peripheral arterial occlusive disease. The resultant model deepens understanding of the true relationships and relative predictive value of the circulating cells for major adverse event in ways not possible with traditional null hypothesis testing statistics. The resultant robust averaged model can be used to risk stratify patients for treatment selection and increase patient compliance with lifestyle modifications and medication use. The developed model should be used to guide further research into understanding of the pathogenesis of major adverse events in this population.

Circulating cell counts of monocytes, lymphocytes and neutrophils together with disease severity at presentation, hypertension and age were more important predictors for the outcome of major adverse event than other traditional risk factors using multi-model inference to develop the best fit model for this data set (Table 8.17). Only variables with strong biological reasoning and established *a priori* relationship were included in the multi-modelling analysis[132] and this evidence was established in the null-hypothesis testing sections of this chapter prior to commencing multi-model analysis. Multi-model analysis was selected as the most appropriate method because it is able to identify the best predictors of an outcome in complex systems where controlling for the large number of factors affecting outcome is impractical.[132,386] The

multi-model averaging results provide evidence that the morbidity and mortality of major adverse events among patients with peripheral arterial occlusive disease may be more related to the circulating cells as markers of inflammation than the traditional risk factors of smoking, diabetes mellitus, ischaemic heart disease, stroke and transient ischaemic attack associated with the establishment of the disease.

Disease Severity was established as a strong predictor of major adverse event in this population, being present in all 18 of the top models (Δ_i <2) of the multi-model analysis (Table 8.16). The strongest variable effect in the resultant averaged model was demonstrated from the variable Rest Pain despite the small numbers in this group. Tissue Loss also demonstrated an important effect with a larger standardised coefficient in the averaged model for major adverse event than the traditional risk factors of ischaemic heart disease and smoking. Future analysis could consider combining the Rest Pain and Tissue Loss groups into the clinical entity of critical limb ischaemia to increase the power of this group, potentially establishing the disease severity of critical limb ischaemia as the most important categorical variable for the outcome of major adverse event. These findings are consistent with the published literature that demonstrates the high major adverse event rate in this clinically important subsection of the population with peripheral arterial occlusive disease.[28,108] The strength of Disease Severity as a predictor of major adverse event is also important to consider when reviewing peripheral arterial occlusive disease literature as not all studies report or account for the clinical severity of disease when investigating these patients.[49,222,320] One question not addressed by this research is the mechanism by which Disease Severity influences major adverse event; is it solely as a marker of total atherosclerotic disease burden including in the coronary and cerebral

circulations, or does the ischaemia of peripheral tissue cause inflammation and a change in the circulating cells that is able to effect ischaemic change in distant organs and subsequent major adverse event.

Age and gender (stratified) were also strong predictors of major adverse event in the multi-model analysis, present in all 18 of the best fit models (Δ_i <2). Age is the only continuous variable in the analysis and although has a small relatively small standardised coefficient in the averaged model (0.06 ±0.01, Table 8.17) generated the largest z value due to the continuous nature of this variable. Gender required stratification due to skewed distribution to ensure reliable inference from the final averaged model and therefore does not have a standardised coefficient presented in Table 8.17. Age and gender are important to include in the resultant averaged model and should be included in any further investigations of this population.

The circulating cells of neutrophils, lymphocytes and monocytes were present in more than half of the best fit models for major adverse event and should be considered consistent predictors for the outcome of major adverse event (Table 8.16). The High Monocyte Category and High Neutrophil Category demonstrated a positive association with the endpoint of major adverse event while both High Lymphocyte Category and Mid Lymphocyte Category had a negative or protective association with this outcome. The circulating cells relationship with the outcome of major adverse event will be addressed individually, with comparison to the null hypothesis testing precedent for inclusion in multi model analysis in previously published literature.

High Monocyte Category was the strongest cell type predictor of major adverse event with the largest standardised coefficient of the circulating cells in the averaged model (0.56 ±0.43) although with a larger standard error than the other circulating cell count categories. The High Monocyte Category was significantly associated with the outcome of major adverse event in all Cox models and clearly established evidence to support Monocyte Category inclusion in the multi-model analysis. High monocyte count has previously been associated with the presence of peripheral arterial occlusive disease in population studies[264] and major adverse event in patients with ischaemic heart disease[99] and has been previously reported to be associated with major adverse event in patients with peripheral arterial occlusive disease.[69] High Monocyte Count has been associated with the early development of atherosclerotic disease[387-390]and although Monocytes are believed to play a critical role in the initiation of atherosclerotic plaque development, they have also been implicated in promoting plaque instability and remodelling after heart attack.[391] When the Cox proportional hazards comprehensive risk factor adjusted model was stratified for other significant categorical variables (smoking, ischaemic heart disease and hypertension) the hazard ratio indicated a 1.75 greater risk of major adverse event with a high monocyte count. This was greater than the upper quartile monocyte category relative risk of major adverse event 1.24 (1.08–1.41, 0.01) found by Grau et al.[69] although they comment that their study may have underestimated risk by 30-35% due to regression dilution. In the current study, the non-stratified Cox model adjusted for traditional risk factors alone, the Cox.zph value reached the 0.05 level indicating a possible breach in the proportional hazards assumption of the model. This was not the case with the comprehensive risk factor adjusted models and the strong positive association of High Monocyte count with major adverse event is a sound and important basis for inclusion in the

multi-model analysis. The increase in circulating monocyte count associated with major adverse event is potentially a result of increased mobilisation of these cells from the marginal pool caused by tissue ischaemia.[392] This study was not designed to investigate monocyte subtypes although the CD14+/CD16+ monocyte subset have previously been shown to contribute to inflammation through their production of tumour necrosis factor – alpha (TNFα).[393] Replication of this study in a larger population using multi-model analysis could be powered to analyse subtypes and may reduce the resultant standard error of this variable and establish monocytes as an important direction for further research in the peripheral arterial occlusive disease population.

The strong protective effect of both High Lymphocyte Category and Mid Lymphocyte Category is demonstrated in this study through negative coefficients in the averaged model (-0.53 ±0.30, -0.49 ±0.28 respectively, Table 8.17). This finding confirms the direction and strength of association demonstrated on Cox proportional hazards testing but also quantifies the relative strength of each Lymphocyte Category with relation to the contribution of other variables to the averaged model. High Lymphocyte Category and Hypertension have the same negative standardised co-efficient or similar protective effect for the outcome of major adverse event, an effect thirteen times that of traditional risk factor smoking (Table 8.17). Lymphocytes have been rarely demonstrated to be significant contributors to the outcome of major adverse event in published literature with significant univariate analysis results of the large population with atherosclerosis in the CAPRIE study, although in that study the association was not significant in that multivariate analysis.[69] In null hypothesis testing Cox proportional hazards analysis both Mid and High Lymphocyte categories were significantly associated with the

endpoint of major adverse event in all Cox models except the stratified comprehensive risk factor adjusted model (Table 8.6). This effect was not found in the

CAPRIE study by Grau et al.[69] who reported the upper quartile lymphocyte category non-significant relative risk of major adverse event as 1.04 (0.91–1.19) on multivariate analysis. Caution needs to be employed in reviewing the Cox proportional hazards results from the current study because the Mid Lymphocyte Category had significant Cox.zph scores indicating a breach of the assumption of proportionality. The Low Lymphocyte Category in the Haumer et al.[72] study of 398 similar patients, showed a trend to significance with the lower tertile lymphocyte count that was not significant (0.07) although the Cox.zph scores were not reported. Tests of proportionality are not commonly published in studies assessing outcome in patients with peripheral arterial occlusive disease, and as the aim is to investigate and describe a complex biological system the most appropriate analysis needs to be again considered. While it may be argued that alternative null hypothesis testing statistics may be performed, the significance of these findings and the potential breach of the assumption of proportionality was sufficient to include Lymphocyte Category in the more appropriate multi-model analysis. Information-Theoretical approaches and model averaging have been proposed to be methodologically superior to traditional null hypothesis testing when variables are correlated.[132] It is possible that the use of the multi-model analysis has unmasked the protective effect of the Lymphocyte category that in null hypothesis testing multivariate analysis and stepwise regression was being clouded by a weaker correlated traditional risk factor. The protective relationship of lymphocytes for the outcome of major adverse event requires replication in other populations with peripheral arterial occlusive disease prior to application

of this information in directing pathophysiological research or potential therapeutic exploitation.

High Neutrophil Category demonstrated a stronger positive effect on the endpoint of major adverse event in the averaged model than all of the traditional risk factors which quantifies and adds support to the previously reported effect of neutrophil count adding to the information of traditional risk factors in predicting major adverse event.[72] High Neutrophil Category was significantly associated with the endpoint of major adverse event in all Cox models including the comprehensive risk factor adjusted model that stratified for other significant categorical variables (smoking, hypertension, ischaemic heart disease) with a hazards ratio 1.98 (1.12-3.10). This is consistent with the hazard ratio found by Haumer et al.[72] (hazard ratio 2.20) for upper tertile neutrophil count and major adverse event in patients with peripheral arterial occlusive disease. A lesser hazard ratio was found for Neutrophil count by Giugliano et al.[74] investigating 259 patients with peripheral arterial occlusive disease (hazard ratio 1.29, 1.06–1.56) after adjusting for cardiovascular drugs although this result was still highly significant (0.02). The Mid Neutrophil Category was not significantly associated with the endpoint of major adverse event in either the *a priori* or adjusted Cox models, but note that in the Kaplan-Meir freedom from major adverse event analysis, Mid Neutrophil Category did trend below that of the Low Neutrophil Category although the plots intersected several times throughout the study possibly as a result of sample size (Figure 8.3). The Mid Neutrophil Category demonstrated the weakest effect of the important circulating cell subtypes in the averaged model (0.18 ±0.26, Table 8.17) and the standard error is larger than the standardised coefficient, although this effect remains 4.5 times the effect of the traditional risk factor smoking. High

Neutrophil Category was a better predictor of major adverse event in multi-model analysis and Mid Neutrophil Category may have its predictive value in the model strengthened by replication of this study in a larger population. The mechanism by which high neutrophil count contributes to major adverse event may be through free radical release,[321] aggregation,[394,395] adhesion,[396] obstruction of microvasculature[190,234] or plaque disruption[397] or a combination of all these factors.

The calculated neutrophil-lymphocyte ratio did not feature in any of the 18 top models (with Δ_i <2) for the outcome of major adverse event. The neutrophil-lymphocyte ratio has recently in other studies been associated with the outcome of major adverse event in patients with peripheral arterial occlusive disease.[49,260,263] The current study objectively demonstrates that the information gained in using both the Neutrophil Category and Lymphocyte Category in multi-model analysis is greater than that of the simplified neutrophil-lymphocyte ratio despite the penalty of additional degrees of freedom. This is an important finding as traditional stepwise regression does not allow direct comparison of these variables in their precision[306] of predicting major adverse event in this population. In null hypothesis testing analysis High NLR Category was significantly associated with the outcome of major adverse event in *a priori* and non-stratified adjusted models. In the Cox proportional hazards model which adjusted for comprehensive risk factors there were five other variables that were also significantly associated with the outcome of major adverse event (age, smoker, ex-smoker, ischaemic heart disease and hypertension). When this model was stratified for the significant categorical variables (smoking, hypertension, ischaemic heart disease) the High NLR Category was no longer significantly associated with the outcome of major adverse event at the 0.05 level (0.09)

and the hazard ratio of 1.56 with 95% confidence interval including the value of 1 (0.93-2.61) while age remained significantly associated. Whilst there remains a trend to significance in null hypothesis testing analysis these results raise the possibility of one or more of the confounding factors actually providing the statistical significance for the High NLR Category in other models. This is another illustration of how the variables that are selected for adjustment in Cox proportional hazards analysis can greatly influence outcome. The potential that a confounder is actually providing some of the effect attributed to the High NLR Category in the *a priori* and non-stratified null hypothesis testing approach is another possible reason that NLR Category did not feature in any of the top model set (Δ_i <2) of the multi-modelling approach with other variables more accurately able to describe the association seen in null hypothesis testing. This finding directly demonstrates one flaw of the null hypothesis testing approach where truly less important variables may appear significant. In a biological system such as the one being investigated here with complex interactions between confounding variables many of which have small but important effects, a more accurate and reliable method of statistical analysis needs to be considered and applied. One fundamental difference in these statistical processes is that instead of disproving the null hypothesis, multi-model analysis allows simultaneous comparison of multiple variables involved in creating the model set and is able to quantify the contribution of these variables. The result of this multi-model analysis is clear that inclusion of Neutrophil Category and Lymphocyte Category better predicts the outcome of major adverse event than the calculated Neutrophil-Lymphocyte Ratio.

The strongest effect from a traditional risk factor in the averaged model was the negative association of hypertension with major adverse event (-0.53 ±0.30, Table 8.17). The reason for

this negative association is not clear from the analysed data with hypertension traditionally having a positive association with mortality.[100,103,398] Treatment of hypertension in peripheral arterial occlusive disease has been demonstrated to lower the incidence of major adverse event in large population studies such as the HOPE (Heart Outcomes Prevention Evaluation) study[104,399,400] with similar results in other study subpopulations with peripheral arterial occlusive disease.[125] At recruitment 74.9% of this study population was noted to have a diagnosis of hypertension or be on treatment for hypertension with 42.0% of the total study population taking ACE-inhibitors, 28.6% beta-blockers, 27.9% calcium channel blockers, 22.6% angiotensin II antagonists, 12.6% taking frusemide and 13.1% were taking another diuretic (Table 5.1). The influence of these antihypertensive medications at recruitment and any changes in anti-hypertensive medication that were recorded at each follow up visit, or correlation with non-invasive blood pressure measurements were outside the scope of the current study and are suggested directions for further investigation of the negative predictive value of the risk factor Hypertension.

Intermittent claudication was also negatively associated with the outcome of major adverse event in the averaged model indicating a protective effect of this disease severity at presentation (-0.39 ±0.54, Table 8.17). The small magnitude of the negative averaged coefficient and relatively large standard error demonstrate that the direction of this result should be interpreted with caution as the large standard error includes 0. Intermittent claudicants have previously been demonstrated to have a lower risk of cardiovascular event than patients with critical limb ischaemia[28] so whether Intermittent Claudication is truly protective or only relatively

protective compared to more advanced disease severity at presentation would need to be examined in a larger population.

Null hypothesis testing of the TWCC categories demonstrated comparable results to previously published studies although TWCC Category was not included in the multi-model analysis as TWCC is a direct result of the summation of cell subtypes. High TWCC was associated with the endpoint of major adverse event in all Cox proportional hazards models at a $p < 0.01$ and a hazard ratio of 2.07 (1.21-3.54, Table 8.2) in the stratified model that adjusted for comprehensive risk factors. This hazard is greater than that reported by Grau et al.[69] who reported a relative risk of 1.42 (1.25–1.63) in a large study of 18,000 patients with atherosclerotic vascular disease, and Giugliano et al.[74] who found a hazard ratio 1.29 (1.06-1.57) in a smaller study of 259 peripheral arterial occlusive disease patients after adjusting for cardiovascular drugs. A study with exactly the same number of patients with peripheral arterial occlusive disease as this study, did not demonstrate a significant association of TWCC with major adverse event over a median follow up of 20 months ($p=0.22$).[72] The ADEP[73] study of 2111 patients did not use tertiles for TWCC but found an odds ratio of 1.15 for a difference of 2.0×10^9 cells/L which was significant for a larger composite outcome of cerebrovascular event (which included death, heart attack and stroke +TIA, +angina, + severe renal failure, + pulmonary embolism, + DVT, + peripheral obstructive arterial disease deterioration). Amaranto et al.[320] showed a significant association with increasing TWCC prior to major endovascular surgery and major adverse event with an odds ratio of 1.67. Future comparison of TWCC with cell subtypes using multi-model analysis would objectively quantify whether

TWCC or the cell subtypes are better predictors of major adverse event and should be considered in future study and analysis design.

The relationship of Mid TWCC Category with major adverse event on null hypothesis testing analysis was less clear than that of High TWCC Category. Mid TWCC Category was significantly associated with the endpoint of major adverse event in *a priori* and comprehensive risk factor adjusted Cox models but was not associated with the endpoint of major adverse event when adjusted for traditional risk factors alone. This raises suspicion that one of the comprehensive risk factors (disease severity, aspirin or statin use) that were not adjusted for in the traditional risk factor analysis, plays an important role and may mask association with the endpoint of major adverse event. In the comprehensive risk factor adjusted Cox model: age, ischaemic heart disease, hypertension, smoking and gender were all significantly associated with major adverse event as well as Mid TWCC (0.01, Table 8.2). When the comprehensive risk factor adjusted Cox model was stratified for the significant categorical variables Mid TWCC remained significantly associated with the outcome of major adverse event (0.02, hazard ratio 1.87, Table 8.2). These results clearly display the underlying complex interaction of Mid TWCC Category and the interplay of risk factors for the outcome of major adverse event. This is one example demonstrating the importance of the publication of all statistical analysis that were implemented and not merely the reporting of significant results. In this case one of the traditional risk factors of age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking or diabetes mellitus is interacting in a way that affects the null hypothesis testing of the association of Mid TWCC Category and the outcome of major adverse event. This is unlikely to be caused by ischaemic heart disease alone as stratifying for

this variable did not change the hazard ratio or p value (Table 8.2). Adjusting for the comprehensive risk factors in Cox analysis not only produced a significant result for Mid TWCC Category but also revealed six of the risk factors to have a significant association with the outcome of major adverse event. Although all of these variables were present in the previously described analysis, interaction with Disease Severity, Aspirin or Statin use revealed a different profile of significant factors. When the Cox proportional analysis was then stratified for these significant factors Mid TWCC Category remained significantly associated with the outcome of major adverse event. The interpretation of one of these results in isolation would omit important information on the interactions of the risk factors and TWCC for the outcome of MAE and unfortunately in simplifying the presentation of results for publication the complexity of these relationships are often understated.

Mid Haemoglobin Category was significantly associated with the outcome of major adverse event in the *a priori* Cox proportional hazards model only, while the High Haemoglobin Category trended to significance (0.06, Table 8.10) and justified the inclusion of this variable in the multi-model analysis. Neither Haemoglobin Category remained significant when the model was adjusted for traditional or comprehensive risk factors suggesting that one or more of the confounding factors were truly responsible for the apparent difference seen on *a priori* modelling. This data set demonstrates the importance of considering and adjusting for potential confounding factors that may mask or exaggerate the true relationship of a variable with the tested outcome. Whilst Haemoglobin has been associated with the outcome of death in populations of patients with peripheral arterial occlusive disease[49,288] there is a paucity of evidence for Haemoglobin association with the outcome of major adverse event. This finding

was confirmed by the total exclusion of Haemoglobin Category from the top model set (Δ_i <2) in the multi-model analysis for the outcome of major adverse event in this study. Multi-model analysis in this study demonstrates that Haemoglobin category is not a good predictor of major adverse event and need not be included in future analysis for this outcome.

The comparative nature of multi-model analysis does not directly compare pathophysiological causality of investigated variables with the outcome of major adverse event. However, it quantifies the relative ability of these variables to discriminate the patients with established peripheral arterial occlusive disease that will sustain a major adverse event which may be very different to the risk factors that cause the establishment of the disease. All categories of disease severity, High and Mid Monocyte Category, High and Mid Lymphocyte Category, High and Mid Neutrophil Category, Hypertension and Ischaemic heart disease, all demonstrated stronger effects in the averaged model for major adverse event than other traditional risk factors of transient ischaemic attack, smoking, statin use and diabetes mellitus. These results are consistent with the early findings of Dormandy and Murray[24] who reported the absence of predictive value for the traditional risk factors of hypertension, diabetes, smoking and high plasma cholesterol levels and suggested that these risk factors for the development and early progression of atherosclerosis are not necessarily prognostic factors in the final stages of the disease.[24]

While the averaged model may seem complicated for the clinician to use at the bedside, a simple computational application for smartphone is proposed to make the model more clinician friendly. With smart phones now commonplace among medical professionals,[401,402] an

application with simple interface that requires selecting the patient risk factors in a yes/no toggle and category of circulating cell count via a high / medium / low slide (with values provided for each category) the clinician would be able to apply this model with nothing more than clinical history and full blood count results. Validation of these results among other populations of patients with peripheral arterial occlusive disease is considered essential prior to this application development. Multi-model inference analysis results show that patient age, gender, clinical disease severity, history of hypertension or ischaemic heart disease should be combined with Lymphocyte Category, Neutrophil Category, and Monocyte Category in the model to predict major adverse events in patients with peripheral arterial occlusive disease. Smoking status, history of Transient Ischaemic Attack, Diabetes Mellitus and Statin use would best have their role in the model clarified by replication with another population, however from this study alone they add meaningfully to the model and should be included. This proposed handheld application may assist the clinician to predict and communicate risk of major adverse event to the patient in a way that may enhance adherence to lifestyle changes and medication use[133] that have been shown to improve outcomes in patients with peripheral arterial occlusive disease,[83,85,135,136] and may help select the most appropriate treatment for individual patients.[137]

Future investigations should replicate this study design with multi-model analysis of larger data sets in similar populations of patients with symptomatic peripheral arterial occlusive disease to refine the predictive ability of the model. Multi-model comparison to compare TWCC with subtypes for the outcome of major adverse event should be considered. Further investigation to define the protective effect of hypertension or the treatment applied for hypertension within this population may give further insight and add strength to the known

benefits of management of this risk factor in peripheral arterial occlusive disease patients.[400] Future multi-model analysis could also be designed to quantitatively compare Ankle Brachial Index and clinical disease severity categories in their ability to predict major adverse event. Expansion of the study population to the wider population of peripheral arterial occlusive disease patients including asymptomatic patients and subsequent model development could aid in identifying asymptomatic patients at the highest risk of major adverse event who would benefit most from the lifestyle changes and medical management discussed above. Using multi-model averaging to create these models for outcome of major adverse event will address the multiple complex interactions of variables demonstrated to complicate null hypothesis testing analysis and will allow comparison of the variables and their association with the endpoint of major adverse event, these results may also be used to infer the key underlying biological processes at work.[132,386]

8.5. Conclusion for MAE

Multi-model averaging is a relatively new statistical paradigm. It is the preferred statistical method for analysing data from complex biological systems needing to account for multiple co-variables of modest effect that interact[132] to produce clinically relevant outcomes including major adverse events for patients with peripheral arterial occlusive disease. Multi-model averaging in this study has demonstrated that circulating cell counts of monocytes, lymphocytes and neutrophils together with disease severity at presentation, hypertension and age are more important in models to predict the outcome of major adverse event than other traditional risk factors.

Rest Pain was the strongest predictor of major adverse event in the best averaged model and as previously established in Chapter 7, disease severity should continue to be reported and accounted for when examining outcomes in patients with peripheral arterial occlusive disease.

High Monocyte Category exerted the strongest effect of the circulating cell types almost 1.5 times the effect of ischaemic heart disease in the averaged model for the major adverse event outcome. However strong corroborating evidence for the association of monocytes with the composite outcome of major adverse event in peripheral arterial occlusive disease patients is lacking.

Both High and Mid Lymphocyte Categories were shown to have a strong protective effect on the outcome of major adverse event when analysed with multi-model inference. Despite this effect being reported in large studies[69] it has not been demonstrated to be significant on

multivariate analysis possibly because of interactions with one or more of the traditional risk factors as suggested by the significant Cox.zph scores in the null hypothesis testing sections of this chapter. The Information-Theoretical approach has been suggested as superior when variables are correlated[403] and has objectively revealed High Monocyte Category to be as important as the traditional risk factor of Hypertension and superior to all other traditional risk factors in the prediction of major adverse event. This result previously unavailable with null hypothesis testing statistics of an important protective effect of Lymphocytes for major adverse event in peripheral arterial occlusive disease patients deserves further research.

Analysis using Information-Theoretical framework has shown that the inclusion of Lymphocyte Category and Neutrophil Category together provide better modelling of the outcome major adverse event than the combined Neutrophil-Lymphocyte ratio. This study is the first to use the Information-Theoretical approach to directly compare the strength of variables contribution to models of outcome through quantifying the approach of best model selection. Caution should be employed in further investigation of the comparatively less informative Neutrophil-Lymphocyte ratio in patients with peripheral arterial occlusive disease.

The strong protective effect of Hypertension or the treatment of hypertension in this study is demonstrated to have the largest and most consistent effect of the traditional risk factors. Although this study has not investigated the relative benefits of antihypertensive treatment this has been identified as an interesting area of further research and is suggested to be assessed using the relatively more informative statistical approach of multi-model inference.

Multi-model inferencing should be applied to larger data sets of patients with peripheral arterial occlusive disease to validate the results of this study and provide valuable information about the association of cell counts with outcomes in this population. Once the nature of these associations is refined, more accurate models will be generated to predict the likelihood of major adverse events for individual patients and thus aid patient compliance with lifestyle change and medical therapy and guide treatment selection. It will also enable the generation of future pathophysiological research and treatment options.

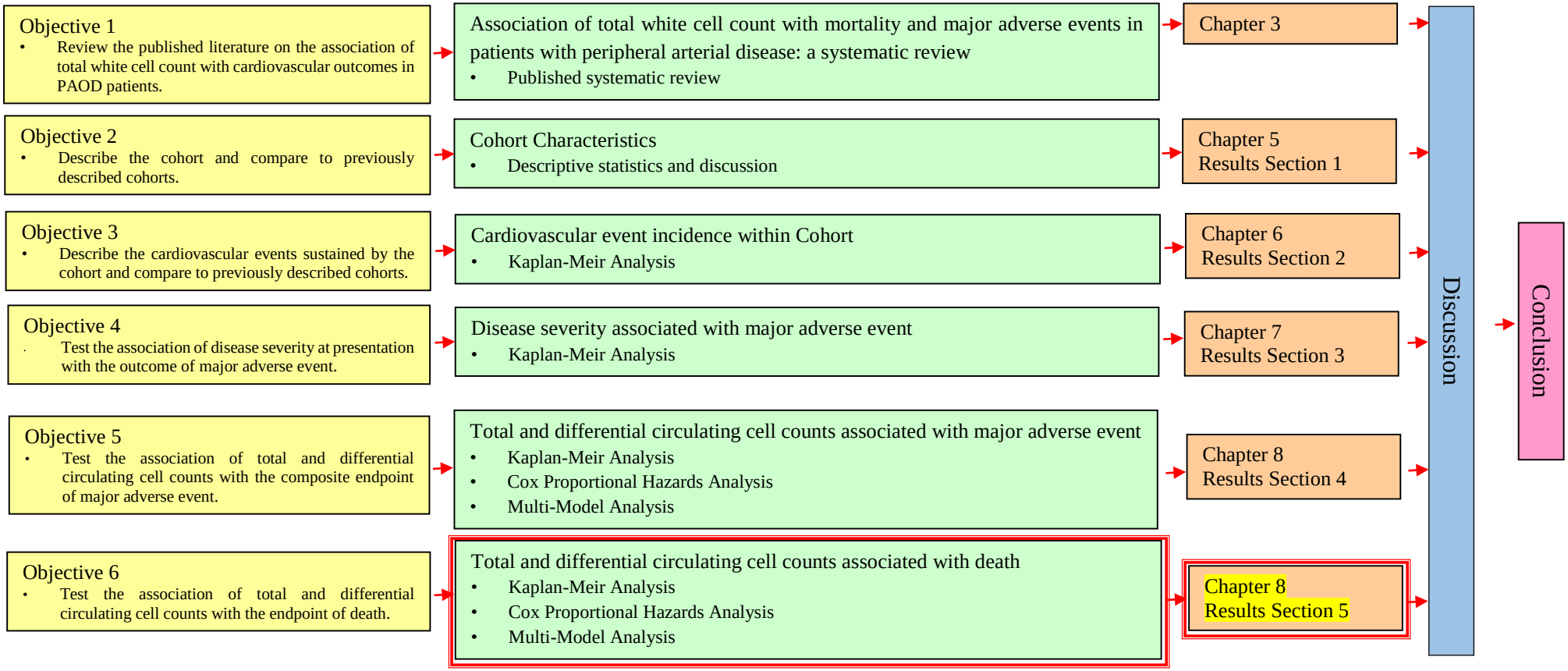

Figure 8.8: Schematic overview of thesis with red box highlighting current position within document – Circulating cell counts associated with death

8.6. Results section 5

Total and differential circulating cell counts associated with death in patients with peripheral arterial occlusive disease

Each circulating cell type is examined and presented in turn for association with death using Kaplan-Meier survival analysis and Cox proportional hazards analysis with and without adjustment for traditional and comprehensive risk factors. The multi-model averaging process is presented at the end of this section to assess all circulating cell types and comprehensive risk factors together in modelling the outcome of death and will report which models and subsequently which variables are best associated with the outcome of death.

8.6.1. Total white cell count associated with death

8.6.1.1. Kaplan-Meier survival analysis

Kaplan-Meier freedom from death plot of the cohort is presented in Figure 8.9 by Total White Cell Count (TWCC) Category. Tertile calculation has been previously explained in Chapter 4 with values available in Chapter 8 Section 4. The horizontal axis is displayed with time points described in Chapter 4.

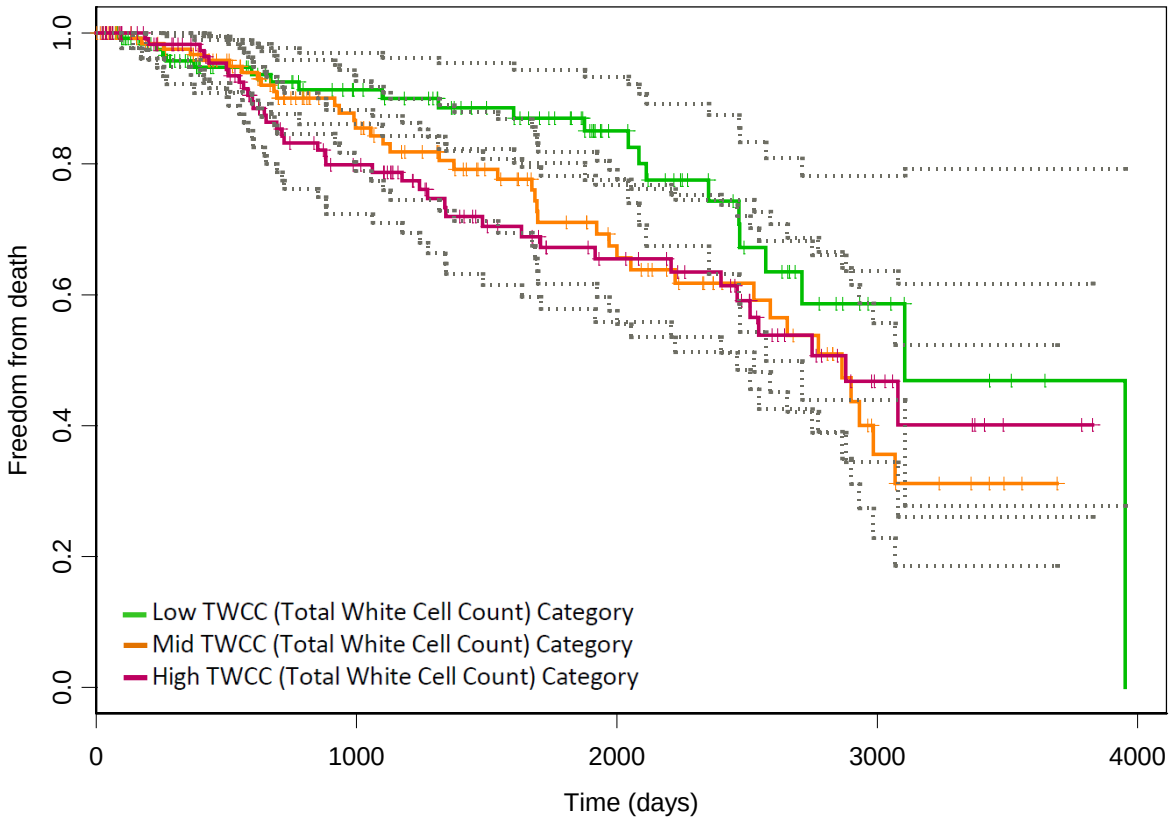

Figure 8.9: Kaplan-Meier nonparametric survival plot - freedom from death by total white cell count category with 95% confidence intervals.

After the first 500 days the Low TWCC Category (green, Figure 8.9) separates and remains above the other two categories for the remainder of the study. The Mid TWCC Category (orange, Figure 8.9) and High TWCC Category (red, Figure 8.9) have multiple visual points of intersection between 2000 and 3000 days (Figure 8.9). The terminal drop off in Figure 8.9 of the Low TWCC Category is due to the last remaining patient dying at that time with all the other groups having the final patients discharged from follow up.

The Mid TWCC Category mean (2511 ±131 days = 6.9 years) and median (2864 days = 7.8 years) survival and the High TWCC mean (2550 ±153 days = 7.0 years) and median (2879 days = 7.8 years) survival are very similar with a similar number of deaths (39, 38 respectively, ~29% of the patients in each category) in both these groups. The Low TWCC category has the greatest mean (2984 ± 171 days = 8.2 years) and median (3105 days = 8.5 years) freedom from death and the least deaths (23, 17.6% of the patients in this category).

Table 8.18: Kaplan-Meier freedom from death rate by total white cell count category with 95% confidence intervals displayed by days follow up.

Death		0 days	1000 days	2000 days	3000 days	3500 days
Low TWCC Category	At Risk	131	70	35	7	3
	Events in previous interval	0	9	4	8	1
	Freedom from death (95% CI)	1.00	0.91 (0.73-0.82)	0.85 (0.78-0.99)	0.59 (0.44-0.78)	0.47 (0.29-0.79)
Mid TWCC Category	At Risk	131	70	35	7	3
	Events in previous interval	0	15	13	10	1
	Freedom from death (95% CI)	1.00	0.86 (0.79-0.93)	0.66 (0.56-0.78)	0.36 (0.23-0.56)	0.31 (0.19-0.52)
High TWCC Category	At Risk	136	75	36	9	2
	Events in previous interval	0	20	10	7	1
	Freedom from death (95% CI)	1.00	0.80 (0.72-0.88)	0.66 (0.59-0.77)	0.47 (0.34-0.64)	0.40 (0.26-0.62)

CI = Confidence Interval TWCC = Total white cell count

The freedom from death rate is highest in the Low TWCC Category at each of the time points reported (Table 8.18). The number of deaths in the Mid TWCC and High TWCC Categories is higher at both 1000 and 2000 days resulting in lower freedom from death rates in these groups at these time points. The High TWCC Category has a greater drop in freedom from death rate prior to 2000 days but is overtaken by the Mid TWCC Category during the latter half of the study indicating crossing of the survival curves after 2000 days. As these groups appear to be

behaving differently they are analysed with Cox proportional hazards model to quantify differences and allow adjustment for confounding factors with results presented in Table 8.19.

8.6.1.2. Cox proportional hazards

Table 8.19: Cox proportional hazards analysis - death and total white cell count category.

Outcome - Death						
Cell Type		p Value	HR (95% CI)	Other Significant Variables	Cox zph	Global zph
TWCC *a priori*	mid	0.07	1.61 (0.96-2.72)	NA	0.79	0.33
	high	**0.05**	**1.68 (0.99-2.84)**		0.34	
TWCC adjusted TRF[1]	mid	0.13	1.50 (0.89-2.55)	Age (p<0.01)	0.88	0.18
	high	**0.04**	**1.75 (1.02-3.02)**		0.48	
TWCC adjusted CRF[2]	mid	**<0.01**	**2.38 (1.35-4.21)**	Age (p<0.01), IHD (p=0.02), Tissue Loss (p=0.02)	0.66	0.23
	high	**<0.01**	**2.09 (1.19-3.67)**		0.80	
WCC adjusted CRF[2] strata (IHD & Disease severity)	mid	**0.02**	**2.00 (1.12-3.55)**	Age (p<0.01)	0.85	0.68
	high	**0.02**	**1.95 (1.11-3.41)**		0.75	

CI = Confidence Interval TWCC = Total white cell count IHD = Ischaemic heart disease
[1] = adjusted for entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
[2] = adjusted for entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
strata = statistical stratification for (variable/s in brackets following)
Significant p values at the 0.05 level and corresponding hazard ratio are presented in **bold**

The High TWCC Category was significantly different to the Low TWCC Category freedom from death rates in all models. Without adjustment for confounding variables (*a priori*) the High TWCC Category was significant at the p<0.05 level (Table 8.19). This significant association remains when the model is adjusted for the confounding variables of the traditional risk factors (adjusted TRF[1], Table 8.19): hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus (p=0.04, Table 8.19). The relationship remains consistently significant with adjustment for the comprehensive risk factors (adjusted CRF[2]: previous traditional risk factors and disease severity and aspirin and statin use) with a significant difference demonstrated in the freedom from death for both the Mid TWCC and High TWCC Category (p<0.01, Table 8.19). In this comprehensive risk factor

(CRF^2) adjusted model for High TWCC Category, the risk factors of ischaemic heart disease and disease severity of tissue loss were also significantly associated with freedom from death. The risk factors of ischaemic heart disease and disease severity were then stratified in the Cox model to determine the individual contribution of High TWCC Category which was again significantly associated with freedom from death (p=0.02, Table 8.19) with the hazard ratio 1.95 (95% CI 1.11-3.41) meaning that the patients in the High TWCC Category were almost twice as likely to die during the follow up period than the patients in the Low TWCC Category.

When the Low TWCC Category and Mid TWCC Category are compared *a priori* (without any adjustment for confounding variables) a non-significant trend was found with p = 0.07, hazard ratio 1.61 (95% CI 0.96-2.72, Table 8.19) with the hazard ratio confidence interval including the value of 1 (no significant difference). When the traditional risk factor confounding variables (adjusted TRF^1, Table 8.19) of hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus are adjusted for, again the difference between these groups was not statistically significant (p=0.13, Table 8.19). However, when Mid TWCC Category was adjusted for comprehensive risk factors (adjusted CRF^2, Table 8.19) of traditional risk factors plus disease severity and aspirin and statin use, a significant difference is demonstrated in the freedom from death of the two groups (p=0.01, Table 8.19) with the hazard ratio of Mid TWCC Category 2.38 (95%CI 1.35-4.21, Table 8.19). In this second comprehensive risk factor (CRF^2) adjusted model for Mid TWCC Category the risk factors of ischaemic heart disease and disease severity of tissue loss were also significantly associated with freedom from death. The risk factors of ischaemic heart disease and disease severity were then stratified in the Cox model to determine the individual contribution of Mid TWCC Category which was again significantly associated with freedom from death (p=0.02) with a

hazard ratio of 2.00 (95% CI 1.12-3.55, Table 8.19) indicating that the patients in the Mid TWCC Category were twice as likely to die during the follow up period of this study than their counterparts in the Low TWCC Category.

The probability of a violation of the assumptions of the Cox proportional hazards model was checked with the Cox.zph function for each model. The Cox.zph function tests the proportionality of each predictor in the model by creating interactions with time and comparing the residuals. There was a low probability of breach of the assumptions of the Cox model for TWCC category and death models with Cox.zph testing results $p = 0.23 – 0.88$ (Table 8.19). With a low probability of assumption breach the Cox proportional hazards models are appropriate to use for this data set and generate valid results.

8.6.2. Neutrophil count associated with death.

8.6.2.1. Kaplan-Meier survival analysis

Kaplan-Meier freedom from death plot of the cohort is presented in Figure 8.20 by Neutrophil Category.

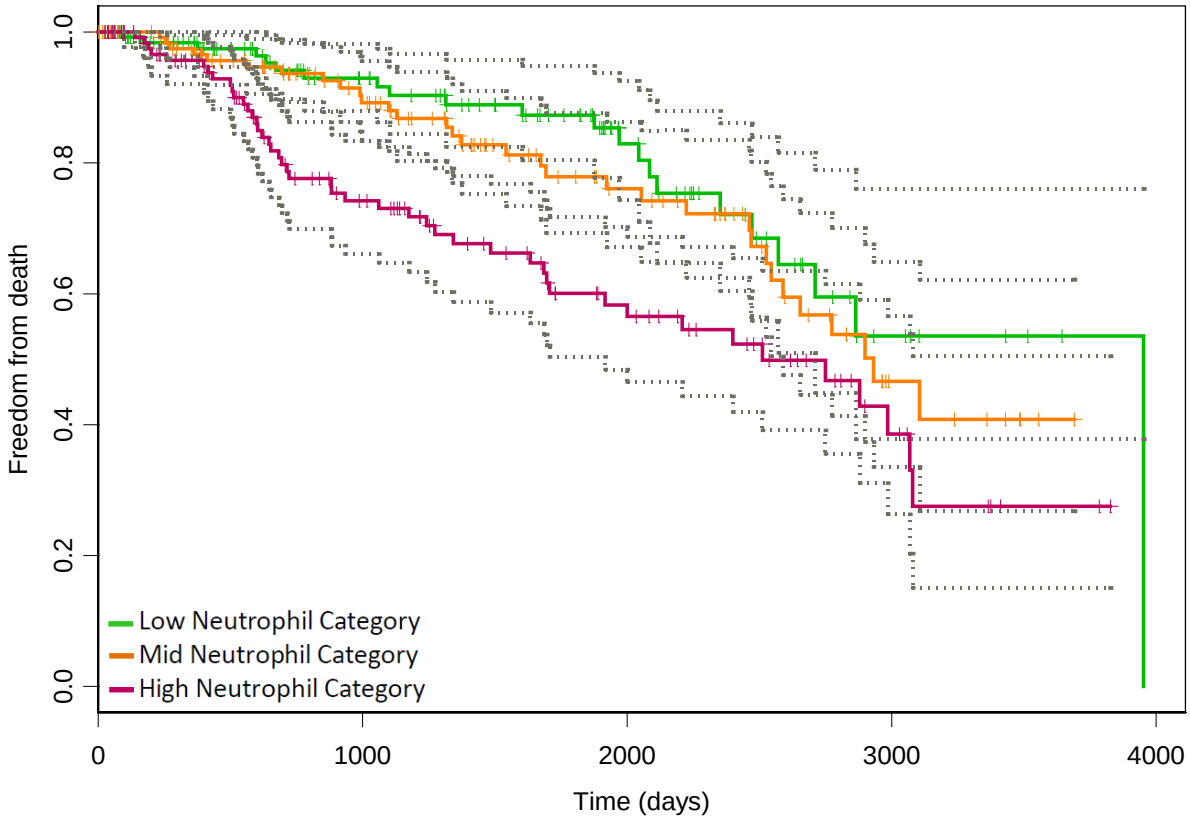

Figure 8.20: Kaplan-Meier nonparametric survival plot - freedom from death by neutrophil count category with 95% confidence intervals.

The High Neutrophil Category (red, Figure 8.20) diverges from the other two neutrophil categories with a consistently lower freedom from death rate. The Mid Neutrophil Category (orange, Figure 8.20) is mostly between the other two groups but has at least four points of intersection with the Low Neutrophil Category (green, Figure 8.20). The terminal drop in

freedom from death in the Low Neutrophil category in Figure 8.20 is due to the last patient in this category dying at this time. The last remaining patients in both other neutrophil categories were discharged from follow up without dying.

The Low Neutrophil Category has the longest duration mean (3041 ±162 days = 8.3 years) and median (3592 days = 9.8 years) freedom from death with the least events (22, 16.2% of the patients in the Low Neutrophil Category). High Neutrophil Category has the shortest mean (2295 ±150 days = 6.3 years) and median (2511 days = 6.9 years) freedom from death and the greatest number of deaths per group (46, 35.4% of the patients in the High Neutrophil category). The Mid Neutrophil Category has a mean freedom from death (2717 ±128days = 7.4 years), median freedom from death (2931 days = 8.0 years) and total number of deaths (32, 24.2% of the patients in the Mid Neutrophil Category) approximately in the middle of the other two groups.

Table 8.20: Kaplan-Meier freedom from death rate by neutrophil count category with 95% confidence intervals displayed by days follow up.

Death		0 days	1000 days	2000 days	3000 days	3500 days
Low Neutrophil Category	At Risk	136	72	34	7	3
	Events in previous interval	0	7	6	8	0
	Freedom from death (95% CI)	1.00	0.93 (0.88-0.98)	0.83 (0.74-0.93)	0.54 (0.38-0.76)	0.54 (0.38-0.76)
Mid Neutrophil Category	At Risk	132	79	41	8	2
	Events in previous interval	0	11	9	11	1
	Freedom from death (95% CI)	1.00	0.89 (0.83-0.96)	0.76 (0.67-0.86)	0.47 (0.34-0.65)	0.41 (0.27-0.62)
High Neutrophil Category	At Risk	130	64	33	9	2
	Events in previous interval	0	26	12	6	2
	Freedom from death (95% CI)	1.00	0.74 (0.66-0.83)	0.57 (0.47-0.69)	0.39 (0.26-0.57)	0.28 (0.15-0.50)

CI = Confidence Interval

The striking outcome of Table 8.20 is the lower freedom from death rate in the High Neutrophil Category and Mid Neutrophil Category at all reported time points. The freedom from death plot in Figure 8.20 gives additional information with the Mid and Low Neutrophil category sharing similar rates of death prior to 1000 days and between 2000 and 3000 days despite a greater total number of deaths in each period in the Mid Neutrophil Group. The difference in survival curves was then analysed with Cox proportional hazards model to compare Neutrophil Categories for the outcome of freedom from death and allow adjustment for confounding factors (Table 8.21).

8.6.2.2. Cox proportional hazards

Table 8.21: Cox proportional hazards analysis - death and neutrophil category

Outcome - Death						
Cell Type		p Value	HR (95% CI)	Other Significant Variables	Cox zph	Global zph
Neutrophil *a priori*	mid	0.31	1.33 (0.76-2.30)	n/a	0.95	0.20
	high	**<0.01**	**2.28 (1.36-3.83)**		0.18	
Neutrophil adjusted TRF[1]	mid	0.38	1.29 (0.73-2.26)	Age (p<0.01)	0.91	0.14
	high	**<0.01**	**2.15 (1.26-3.66)**		0.19	
Neutrophil adjusted CRF[2]	mid	0.14	1.55 (0.87-2.76)	Age (p<0.01)	0.87	0.15
	high	**<0.01**	**2.49 (1.43-4.32)**		0.41	

CI = Confidence Interval n/a = not applicable
TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
Significant p values at the 0.05 level and corresponding hazard ratio are presented in **bold**

The High Neutrophil Category group had significantly different freedom from death function to the Low Neutrophil category in *a priori* and both adjusted models (adjusted for traditional risk factors TRF[1] and adjusted for comprehensive risk factors CRF[2], Table 8.21). The only other variable significantly associated with freedom from death was age which was significantly associated in adjustment for traditional risk factors with, and comprehensive risk factors ($p<0.01$, Table 8.21). The hazard ratio of 2.49 (1.43-4.32, Table 8.21) for the comprehensive risk factor adjusted Cox model means that the High TWCC Category patients were two and a half times more likely to die over the course of this study than the patients in the Low TWCC Category.

The Mid Neutrophil Category was not significantly different to the Low Neutrophil category for the freedom from death outcome in any of the Cox models (Table 8.21) despite apparent differences at the reported time points in Table 8.20. The hazard ratios for the Mid Neutrophil Category all include 1 confirming the lack of significance compared to the Low Neutrophil Category. The crossing of the Mid and Low Neutrophil Category freedom from death curves

at four points in Figure 8.20 (less than 1000 days and between 2000 and 3000 days) is one explanation for this finding.

There was a low probability of breach of the assumptions of the Cox model for Neutrophil Category and death models with Cox.zph testing results p = 0.14 – 0.95 (Table 8.21). With a low probability of assumption breach the Cox proportional hazards models are appropriate to use for this data set and generate valid results.

8.6.3. Lymphocyte count associated with death.

8.6.3.1. Kaplan-Meier survival analysis

Kaplan-Meier freedom from death plot of the cohort is presented in Figure 8.21 by Lymphocyte Category. The terminal drop off in Low Lymphocyte Category (green, Figure 8.21) is due to the last remaining patient dying at that time with all the other groups having the final patients discharged.

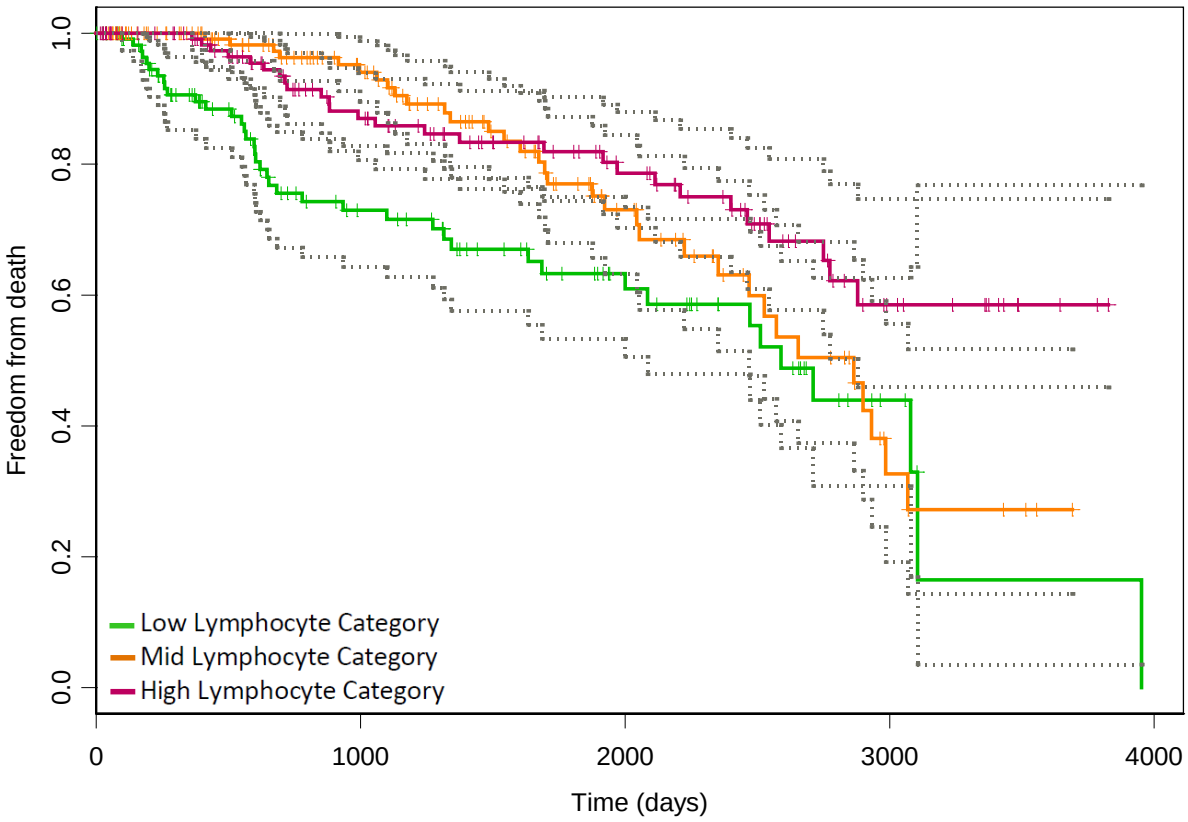

Figure 8.21: Kaplan-Meier nonparametric survival plot - freedom from death by lymphocyte count category with 95% confidence intervals.

The Low Lymphocyte Category plot (green, Figure 8.21) displays an overall lower freedom from death rate than the other two Lymphocyte Categories but does cross over with the Mid

Lymphocyte Category (orange, Figure 8.21) prior to 3000 days. The High Lymphocyte Category (red, Figure 8.21) has the greatest freedom from death rate after 2000 days but prior to 1500 days is below the Mid Lymphocyte Category. The Mid Lymphocyte Category plot (orange, Figure 8.21) displays complex interaction with the other two groups, commencing the study with the greatest freedom from death rate prior to 1500 days, before trending down at a faster rate than the other two categories to fall below the Low Lymphocyte Category plot at 3000 days.

The Low Lymphocyte Category has the most deaths (40, 33.9% of the Low Lymphocyte Category patients) and the shortest mean (2240 ±181 days = 6.1 years) and median (2589 days = 7.1 years) time to death followed by the Mid Lymphocyte Category and the High Lymphocyte Category respectively in all measures of central tendency. The High Lymphocyte Category has the least deaths (27, 20.3% of the High Lymphocyte Category patients) and the longest mean freedom from death (2980 ±136 days = 8.2 years) and a median was not applicable. The Mid Lymphocyte Category was in-between the 2 other groups with 33 events in 147 patients and a mean freedom from death of 2594 ±125 days (7.1 years and a median freedom from death of 2564 days (7.0 years).

Table 8.22: Kaplan-Meier freedom from death rate by lymphocyte count category with 95% confidence intervals displayed by days follow up.

Death		0 days	1000 days	2000 days	3000 days	3500 days
Low Lymphocyte Category	At Risk	118	53	27	5	1
	Events in previous interval	0	25	7	5	2
	Freedom from death (95% CI)	1.00	0.73 (0.64-0.83)	0.61 (0.51-0.73)	0.44 (0.31-0.63)	0.17 (0.04-0.77)
Mid Lymphocyte Category	At Risk	147	83	34	6	3
	Events in previous interval	0	6	14	12	1
	Freedom from death (95% CI)	1.00	0.94 (0.90-0.99)	0.73 (0.63-0.84)	0.33 (0.19-0.56)	0.27 (0.14-0.52)
High Lymphocyte Category	At Risk	133	79	47	13	3
	Events in previous interval	0	13	6	8	0
	Freedom from death (95% CI)	1.00	0.87 (0.81-0.94)	0.79 (0.70-0.88)	0.59 (0.46-0.75)	0.59 (0.46-0.75)

CI = Confidence Interval

The Low Lymphocyte Category has the greatest number of deaths in the first 1000 days (25, Table 8.22) and has a lower freedom from death rate at each reported time point than the High Lymphocyte Category. The Mid Lymphocyte Category has the largest number of deaths in the 1000 to 2000 day period and has a freedom from death rate lower than the High Lymphocyte Category from this point until the end of the study. The Low and Middle Lymphocyte Categories do cross over with their respective freedom from death functions at approximately 3000 days which can be seen in both Figure 8.21 and Table 8.22. The groups were compared with Cox proportional hazard model to allow adjustment for confounding factors with results and are presented in Table 8.23.

8.6.3.2. Cox proportional hazards

Table 8.23: Cox proportional hazards analysis - death and lymphocyte category.

Outcome - Death						
Cell Type		p Value	95% CI)	Other Significant Variables	Cox zph	Global zph
Lymphocyte *a priori*	mid	**0.04**	**0.61 (0.38-0.99)**	n/a	**<0.01**	**<0.01**
	high	**<0.01**	**0.43 (0.26-0.70)**		0.57	
Lymphocyte adjusted TRF[1]	mid	0.06	0.64 (0.40-1.03)	Age (p<0.01)	**0.01**	**<0.01**
	high	**<0.01**	**0.49 (0.29-0.81)**		0.69	
Lymphocyte adjusted CRF[2]	mid	0.22	0.73 (0.44-1.20)	Age (p<0.01), Ex-smoker (p=0.04), IHD (p=0.04), Tissue loss (p=0.07), Smoker (p=0.08), HTN, (p=0.09),	**0.01**	**0.02**
	high	**0.02**	**0.54 (0.31-0.91)**		0.60	
Lymphocyte fully adjusted CRF[2] Strata (smoking, HTN, IHD, Disease severity)	mid	0.21	0.68 (0.37-1.24)	Age (p<0.01)	0.08	0.49
	high	0.10	0.59 (0.32-1.11)		0.44	

CI = Confidence Interval HTN = Hypertension IHD = Ischaemic heart disease
n/a = not applicable Smoker = Current smoker
TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
strata = statistical stratification for (variable/s in brackets following)
Significant p values at the 0.05 level and corresponding hazard ratio are presented in **bold**

Low Lymphocyte Category was significantly different to both Mid Lymphocyte Category and High Lymphocyte category when analysed with the Cox proportional hazards model *a priori* (Table 8.23). The Mid Lymphocyte Category was significantly inversely associated in the *a priori* Cox model with p=0.04 and hazard ratio of 0.61 (0.38-0.99, Table 8.23). With the hazard ratio being less than one, the Mid Lymphocyte Category is at lower risk of death than the Low Lymphocyte Category. The High Lymphocyte Category was also significantly different to the Low Lymphocyte Category *a priori* at the p<0.01 level and hazard ratio 0.43 (0.26-0.70, Table 8.23). The hazard ratio for High Lymphocyte Category is also less than one indicating a lower risk of death or protective function in this category when compared to the Low Lymphocyte Category.

When adjusted for the traditional risk factors of hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus (adjusted TRF[1], Table 8.23) only the High Lymphocyte Category is significantly different to Low Lymphocyte Category ($p<0.01$, Table 8.23).

The Cox model that adjusts for comprehensive risk factors: disease severity and aspirin and statin use as well as traditional risk factors (adjusted CRF[2]), revealed multiple factors to be significant including High Lymphocyte Category. The other significant risk factors in the adjusted CRF[2] model were age, previous smoking, disease severity – tissue loss, current smoking and hypertension. The Cox model adjusted CRF[2] was then stratified for these other significant categorical variables and neither Mid or High Lymphocyte Category was significantly associated with death more than the Low Lymphocyte Category at the $p<0.05$ level, however the High Lymphocyte Category did trend toward an association ($p=0.10$, Table 8.23).

Cox.zph values for the Mid Lymphocyte Category are significant in all non-stratified models for death ($p\leq0.01$, Table 8.23) raising the possibility of an assumption violation of these Cox proportional hazards models. The global zph score is also significant in these models confirming that the overall assumptions of the model are breached by the non-proportionality of the Mid Lymphocyte Category. A visual representation for this non-proportionality was seen in the Kaplan-Meir freedom from death plot (Figure 8.21) with the Mid Lymphocyte Category commencing the study above the other lymphocyte categories and finishing below. This raises

suspicion that the Cox proportional hazards model is not appropriate to use for this data set and may generate invalid results.

8.6.4. Monocyte count associated with death.

8.6.4.1. Kaplan-Meier survival analysis

Kaplan-Meier freedom from death plot of the cohort is presented in Figure 8.22 by Monocyte Category.

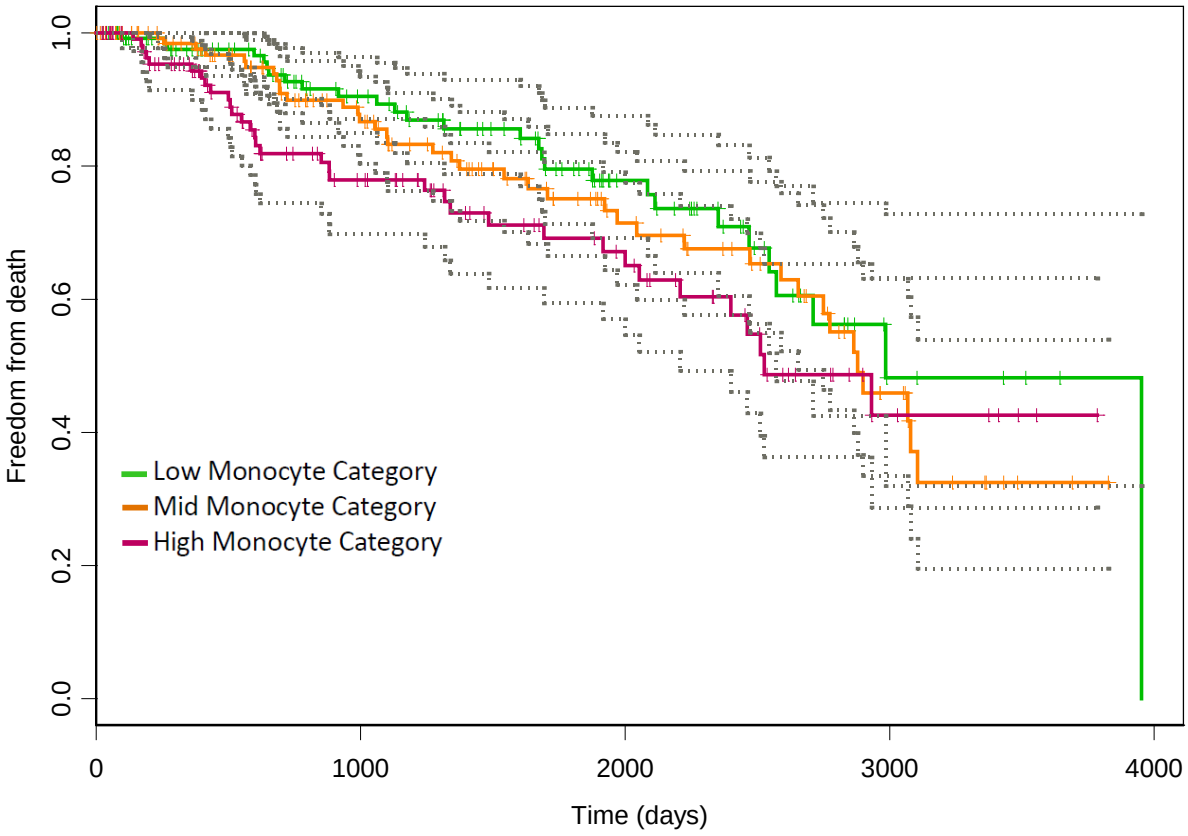

Figure 8.22: Kaplan-Meier nonparametric survival plot - freedom from death by monocyte count category with 95% confidence intervals.

The High Monocyte Category (red, Figure 8.22) displays a lower freedom from death plot than the other two categories with the exception of an intersection with the Mid Monocyte Category (orange, Figure 8.22) prior to 3000 days. The terminal drop in the Low Monocyte Category

(Figure 8.22) is due to the last remaining patient in that category dying at that time, in the other groups the last remaining patients are discharged from follow up.

The High Monocyte Category has the lowest mean (2497 ± 164 days = 6.8 years) and median (2526 days = 6.9 years) freedom from death. The Low Monocyte Category has the highest mean (2922 ±160 days = 8.0 years) and median (2985 days = 8.2 years) freedom from death and the smallest number of deaths (28, 20.9% of the Low Monocyte Category patients). The Mid Monocyte Category sustained the highest number of deaths (38, 26.2% of the Mid Monocyte Category patients) although this category had the largest number of patients (145) giving a mean 2653 ±133 days (7.3 years) and a median of 2879 days (7.9 years)

Table 8.24: Kaplan-Meier freedom from death rate monocyte count category with 95% confidence intervals displayed by days follow up.

Death		0 days	1000 days	2000 days	3000 days	3500 days
Low Monocyte Category	At Risk	134	78	37	5	3
	Events in previous interval	0	10	9	8	0
	Freedom from death (95% CI)	1.00	0.91 (0.86-0.96)	0.78 (0.69-0.88)	0.48 (0.32-0.73)	0.48 (0.32-0.73)
Mid Monocyte Category	At Risk	145	80	39	13	2
	Events in previous interval	0	14	11	10	3
	Freedom from death (95% CI)	1.00	0.87 (0.80-0.94)	0.72 (0.62-0.82)	0.46 (0.33-0.62)	0.33 (0.20-0.54)
High Monocyte Category	At Risk	119	57	32	6	2
	Events in previous interval	0	20	7	7	0
	Freedom from death (95% CI)	1.00	0.78 (0.70-0.87)	0.65 (0.55-0.78)	0.43 (0.29-0.63)	0.43 (0.29-0.63)

CI = Confidence Interval

The High Monocyte Category has the lowest freedom from death at each time point reported (Table 8.24) although there is some interaction of the freedom from death curves prior to the 3000 day time point which is best seen in Figure 8.22. The differences between the monocyte

category associations with death were analysed with Cox proportional hazards model to allow for adjustment of confounding factors and are presented in Table 8.25.

8.6.4.2. Cox proportional hazards

Table 8.25: Cox proportional hazards analysis - death and monocyte category.

Outcome - Death						
Cell Type		p Value	HR (95% CI)	Other Significant Variables	Cox zph	Global zph
Monocyte *a priori*	mid	0.35	1.27 (0.77-2.08)	n/a	0.79	0.10
	high	0.06	1.64 (0.99-2.71)		0.12	
Monocyte adjusted TRF[1]	mid	0.54	1.17 (0.71-1.94)	Age (p<0.01)	0.76	0.07
	high	0.06	1.66 (0.98-2.79)		0.13	
Monocyte adjusted CRF[2]	mid	0.44	1.22 (0.73-2.03)	Age (p<0.01)	0.60	0.07
	high	0.06	1.69 (0.98-2.84)		0.29	

CI = Confidence Interval n/a = not applicable
IHD = Ischaemic heart disease HTN = Hypertension
TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
strata = statistical stratification for (variable/s in brackets following)
Significant p values at the 0.05 level and corresponding hazard ratio are presented in **bold**

High Monocyte Category was not significantly associated with death in either *a priori* or adjusted Cox models as the p values remained slightly above the chosen level of significance of p<0.05 (p=0.06, Table 8.25). The only variable significantly associated with death on adjusting for traditional risk factors (adjusted TRF[1]) and adjusting for comprehensive risk factors (adjusted CRF[2]) was age with a p value <0.01 (Table 8.25). Mid Monocyte Category was not significantly associated with the outcome of death in any of the Cox proportional hazards models (Table 8.25).

There was a low probability of breach of the assumptions of the Cox model for Monocyte category and death models with Cox.zph testing results p = 0.07 – 0.79 (Table 8.25). With a low probability of assumption breach the Cox proportional hazards models are appropriate to use for this data set and generate valid results.

8.6.5. Haemoglobin associated with death.

8.6.5.1. Kaplan-Meier survival analysis

Kaplan-Meier freedom from death plot of the cohort is presented in Figure 8.23 by Haemoglobin Category.

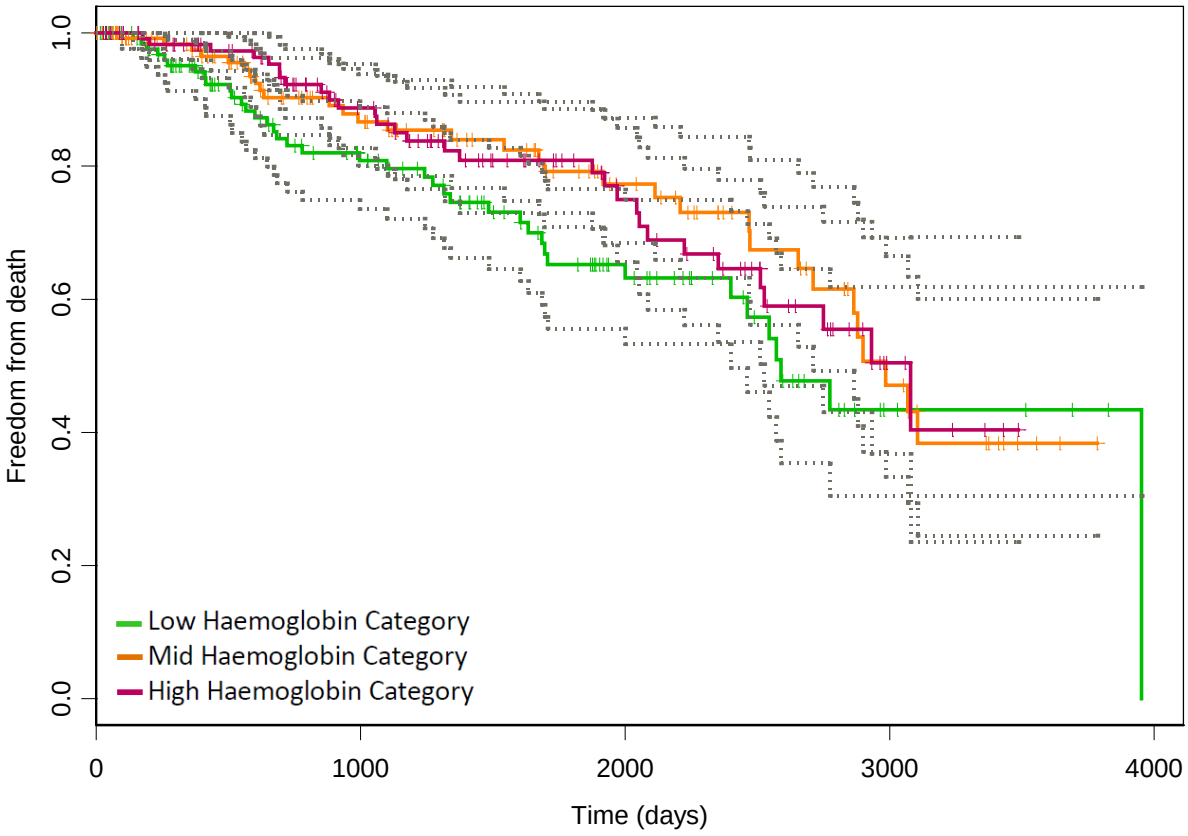

Figure 8.23: Kaplan-Meier nonparametric survival plot - freedom from death by haemoglobin category with 95% confidence intervals.

The Low Haemoglobin Category (green, Figure 8.23) displays a lower freedom from death rate to the other two categories prior to 3000 days. The Low Haemoglobin Category (green, Figure 8.23) has a terminal drop to 0 when the last remaining patient in that category dies, with the other groups having the final patients discharged from follow up without dying. There are

multiple intersections between the Mid Haemoglobin Category plot (orange, Figure 8.23) and the High Haemoglobin Category plot (red, Figure 8.23).

The Low Haemoglobin Category has the greatest number of deaths over the course of the study (39, 28.9% of the Low Haemoglobin Category patients) and has the lowest mean (2594 ± 164 days = 7.1 years) and median (2589 days = 7.1 years) freedom from death. The least deaths occurred in the High Haemoglobin group (30, 23.6% of the High Haemoglobin Category patients) which also had the greatest median freedom from death, taking 3079 days (8.4 years) to have a freedom from death rate less than 0.5. The Mid Haemoglobin Category has the greatest calculated mean freedom from death using the area under the curve calculation (2758 ± 136 days = 7.6 years) although with a median less than the High Haemoglobin Category (2985 days, 8.2years).

Table 8.26 Kaplan-Meier freedom from death rate by haemoglobin category with 95% confidence intervals displayed by days follow up.

Death		0 days	1000 days	2000 days	3000 days	3500 days
Low Haemoglobin Category	At Risk	135	70	32	5	4
	Events in previous interval	0	20	12	6	0
	Freedom from death (95% CI)	1.00	0.81 (0.74-0.89)	0.63 (0.53-0.75)	0.43 (0.31-0.62)	0.43 (0.31-0.62)
Mid Haemoglobin Category	At Risk	136	72	39	13	3
	Events in previous interval	0	13	6	10	2
	Freedom from death (95% CI)	1.00	0.87 (0.80-0.94)	0.77 (0.69-0.87)	0.47 (0.33-0.66)	0.38 (0.25-0.60)
High Haemoglobin Category	At Risk	127	73	37	6	-
	Events in previous interval	0	11	9	9	-
	Freedom from death (95% CI)	1.00	0.89 (0.83-0.95)	0.75 (0.66-0.86)	0.52 (0.37-0.69)	-

CI = Confidence Interval Hb= Haemoglobin

The greatest number of deaths was seen in the Low Haemoglobin Category prior to 2000 days with this group having the lowest freedom from death until the 3000 day time point (Table 8.26). Both the Mid Haemoglobin Category and High Haemoglobin Category intersect the Low Haemoglobin category freedom from death rate between 3000 and 3500 days (Table 8.26 and Figure 8.23). There are multiple points of crossing in the freedom from death function for Mid Haemoglobin Category and High Haemoglobin Category and these are best seen in Figure 8.23. The differences between the haemoglobin category associations with death were analysed with Cox proportional hazards model to allow for adjustment of confounding factors and are presented in Table 8.27.

8.6.5.2. Cox proportional hazards

Table 8.27: Cox proportional hazards analysis - death and haemoglobin category.

Outcome - Death						
Haemoglobin Level		p Value	HR (95% CI)	Other Significant Variables	Cox zph	Global zph
Haemoglobin *a priori*	mid	0.13	0.69 (0.43-1.11)	n/a	0.22	0.36
	high	0.15	0.71 (0.44-1.14)		0.23	
Haemoglobin adjusted TRF[1]	mid	0.44	0.82 (0.49-1.36)	Age (p<0.01)	0.56	0.19
	high	0.93	0.98 (0.56-1.69)		0.89	
Haemoglobin adjusted CRF[2]	mid	0.85	1.08 (0.61-1.84)		0.95	0.13
	high	0.50	1.22 (0.69-2.19)		0.82	

CI = Confidence Interval n/a = not applicable

TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus

CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin

Cox proportional hazard analysis for the endpoint of freedom from death are seen in Table 8.27 comparing both Mid Haemoglobin Category and High Haemoglobin Category to Low Haemoglobin Category. No significant difference was seen between the Haemoglobin categories in either *a priori* or adjusted models using traditional risk factors of hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus (adjusted TRF[1], Table 8.27) or comprehensive risk factors consisting of traditional risk factors, disease severity and aspirin and statin use (adjusted CRF[2], Table 8.27).

There was a low probability of breach of the assumptions of the Cox model for Haemoglobin category and death models with Cox.zph testing results p = 0.13 – 0.95 (Table 8.27). With a low probability of assumption breach the Cox proportional hazards models are appropriate to use for this data set and generate valid results.

8.6.6. Neutrophil-Lymphocyte Ratio associated with death.

8.6.6.1. Kaplan-Meier survival analysis

Kaplan-Meier freedom from death plot of the cohort is presented in Figure 8.24 by Neutrophil-Lymphocyte Ratio (NLR) Category.

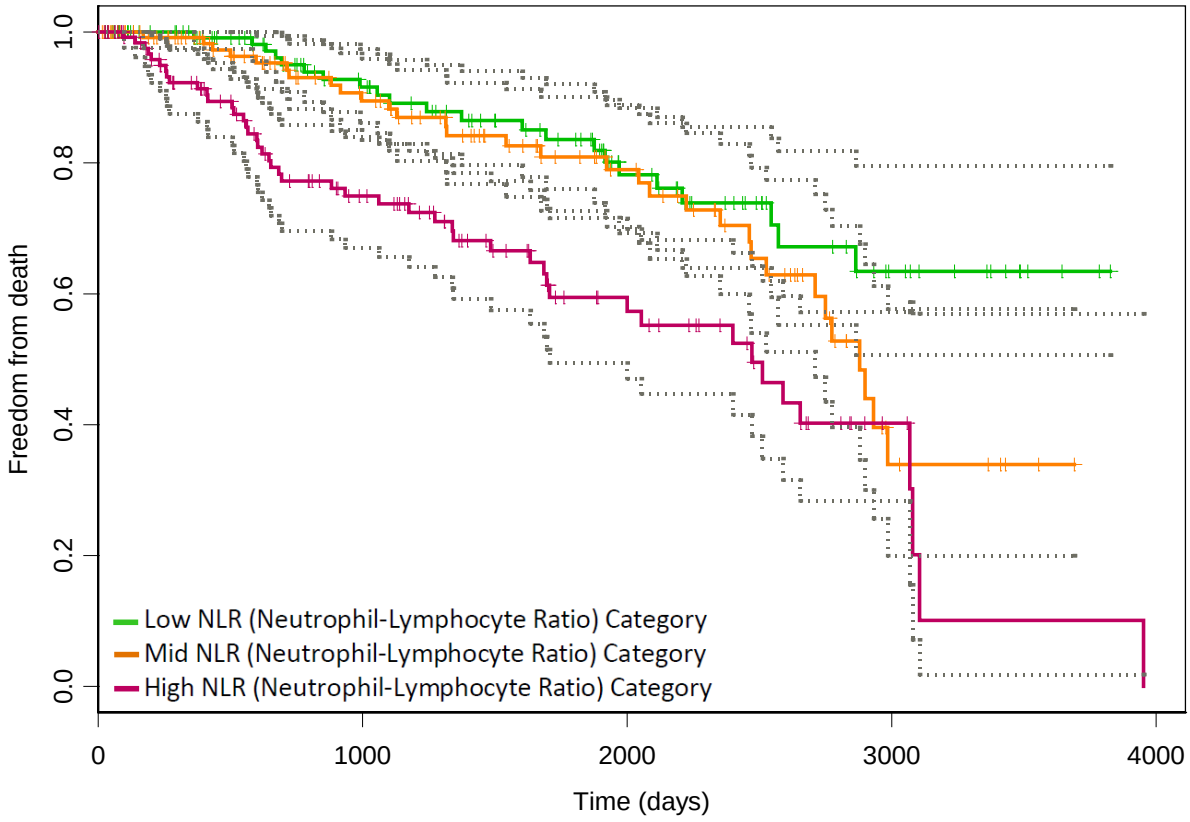

Figure 8.24: Kaplan-Meier nonparametric survival plot - freedom from death by neutrophil-lymphocyte ratio category with 95% confidence intervals.

The High NLR Category plot (red, Figure 8.24) displays a lower freedom from death rate than the other two categories with the exception of an intersection with the Mid NLR Category plot (orange, Figure 8.24) prior to 3000 days. The terminal drop to 0 of the High NLR Category plot is due to the last remaining patient in the High NLR Category dying at that time, with the

other groups having the final patients discharged from follow up. The Low NLR Category plot (green, Figure 8.24) and Mid NLR Category plot (orange, Figure 8.24) are closely related until they diverge after 2000 days with the Mid NLR Category plot dropping to a lower freedom from death.

The greatest number of deaths (47, 35.3% of the High NLR Category patients) was seen in the High NLR Category also having the lowest calculated mean freedom from death using the area under the curve method (2141 ±149 days = 5.9 years). The least number of deaths (22, 16.5% of the Low NLR Category patients) was seen in the Low NLR Category which also had the greatest mean freedom from death (3072 ±136 days = 8.4 years). The Mid NLR Category sustained 31 deaths from a population of 132 patients with mean freedom from death 2679 ±128 days (7.3 years) and a median of 2711 days (7.2 years)

Table 8.28: Kaplan-Meier freedom from death rate by neutrophil-lymphocyte ratio category with 95% confidence intervals displayed by days follow up.

Death		0 days	1000 days	2000 days	3000 days	3500 days
Low NLR Category	At Risk	133	78	40	13	4
	Events in previous interval	0	8	9	5	0
	Freedom from death (95% CI)	1.00	0.92 (0.86-0.97)	0.78 (0.69-0.88)	0.63 (0.51-0.80)	0.63 (0.51-0.80)
Mid NLR Category	At Risk	132	74	40	6	2
	Events in previous interval	0	10	7	14	0
	Freedom from death (95% CI)	1.00	0.90 (0.84-0.96)	0.79 (0.70-0.89)	0.34 (0.20-0.58)	0.34 (0.20-0.58)
High NLR Category	At Risk	127	63	28	5	1
	Events in previous interval	0	26	11	6	3
	Freedom from death (95% CI)	1.00	0.75 (0.67-0.84)	0.57 (0.47-0.70)	0.40 (0.28-0.57)	0.10 (0.02-0.57)

CI = Confidence Interval NLR = Neutrophil-lymphocyte ratio

The Low NLR Category commences the study with 133 patients, of whom eight are deceased prior to 1000 days with 47 patients discharged from follow up during the same period (Table 8.28). From 1000 to 2000 days nine further patients die and 29 are discharged from follow up. Between 2000 and 3000 days five patients from the Low NLR Category die and 22 are discharged from follow up. There are no further deaths in this group between 3000 and 3500 days with 9 patients discharged from follow up in the same period. Of the four patients remaining at 3500 days three are discharged from follow up without event but the last patient remaining in the study in the Low NLR Category dies at 3952 days which is why the freedom from death curve (Figure 8.24) drops to 0 at this point. The Low NLR Category is not distinct from the Mid NLR Category freedom from death function with crossing of their curves at approximately the 2000 day time point. Whether these freedom from death functions were significantly different was tested using the Cox proportional hazards model to allow adjustment for confounding factors and is presented in Table 8.29.

8.6.6.2. Cox proportional hazards

Table 8.29: Cox proportional hazards analysis - death and neutrophil/lymphocyte ratio category

Outcome - Death						
Cell Type		p Value	95% CI)	Other Significant Variables	Cox zph	Global zph
NLR *a priori*	mid	0.12	1.55 (0.90-2.68)	n/a	0.15	0.17
	high	**<0.01**	**2.93 (1.76-4.89)**		0.96	
NLR adjusted TRF[1]	mid	0.33	1.33 (0.75-2.34)	Age (p<0.01), IHD (p=0.42)	0.40	0.13
	high	**<0.01**	**2.24 (1.31-3.81)**		0.80	
NLR adjusted TRF[1] strata (IHD)	mid	0.26	1.39 (0.78-2.46)	Age (p<0.01)	0.28	0.12
	high	**<0.01**	**2.25 (1.32-3.84)**		0.78	
NLR adjusted CRF[2]	mid	0.18	1.49 (0.83-2.69)	Age (p<0.01), IHD (p=0.02), Tissue loss (p=0.04), Ex-smoker (p=0.05), Smoker (p=0.05),	0.36	0.07
	high	**<0.01**	**2.13 (1.23-3.68)**		0.89	
NLR adjusted CRF[2] strata (Smoking, IHD & Disease severity)	mid	0.36	1.34 (0.72-2.48)	Age (p<0.01)	0.35	0.51
	high	**0.04**	**1.84 (1.02-3.31)**		0.94	

CI = Confidence Interval NLR = Neutrophil-lymphocyte ratio n/a = not applicable
IHD = Ischaemic heart disease Smoker = Current smoker
TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin
strata = statistical stratification for (variable/s in brackets following)
Significant p values at the 0.05 level and corresponding hazard ratio are presented in **bold**

The High NLR Category was significantly different to Low NLR Category in all Cox proportional hazards models. In the *a priori* model the High NLR Category had the greatest hazard ratio of 2.93 (95% CI 1.76-4.89, Table 8.29). The High NLR Category was significantly different in all adjusted models with adjustment for traditional risk factors (adjusted TRF1: hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus) and comprehensive risk factors (adjusted CRF[2:] traditional risk factors, disease severity and aspirin and statin use, Table 8.29). When the initial adjusted model revealed other variables to be significantly associated with death, they were stratified in the model to account for their contribution to the outcome of death. With this stratification in the comprehensive risk

factor adjusted model the High NLR category was still significantly associated with death (p=0.04, Hazard ratio 1.84, Table 8.29).

There was a low probability of breach of the assumptions of the Cox model for Neutrophil/Lymphocyte Ratio category and death models with Cox.zph testing results p = 0.07 – 0.94 (Table 8.29). With a low probability of assumption breach the Cox proportional hazards models are appropriate to use for this data set and generate valid results.

8.6.7. Summary of circulating cell types Cox proportional hazards analysis for death

All previously displayed single circulating cell type Cox proportional hazard models for the outcome of death are summarised in Table 8.30.

Table 8.30: Single cell type Cox proportional hazards analysis summary - death.

Death						
Cell Type		*a priori*	Adjusted TRF	Adjusted TRF strata	Adjusted CRF	Adjusted CRF strata
TWCC Category	mid	#	NS	n/a	**	*
	high	*	*	n/a	**	*
Neutrophil Category	mid	NS	NS	n/a	NS	n/a
	high	**	**	n/a	**	n/a
Lymphocyte Category	mid	*	#	n/a	NS	NS
	high	**	**	n/a	*	#
Monocyte Category	mid	NS	NS	n/a	NS	n/a
	high	#	#	n/a	#	n/a
Haemoglobin Category	mid	NS	NS	n/a	NS	n/a
	high	NS	NS	n/a	NS	n/a
NLR Category	mid	NS	NS	NS	NS	NS
	high	**	**	**	**	*

NS = Not significant
n/a = not applicable
* = p value = 0.01 to 0.05
# = p value approaching significance = 0.1 to 0.05
** = p value <0.01
TWCC = Total white cell count
NLR = Neutrophil-lymphocyte ratio
strata = statistical stratification for other significant categorical variables
TRF[1] = traditional risk factors: entry age, gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus
CRF[2] = comprehensive risk factors: entry age, gender hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking, diabetes mellitus, disease severity, aspirin and statin

The level of significance for the circulating cell type in the various Cox proportional hazards models (Table 8.30) depends in all cases on the variables adjusted for and the statistical method of dealing with statistically significant co-variables. The most consistently significantly associated across all models are the High TWCC Category, the High Neutrophil Category and the High NLR Category which are significantly associated with the outcome of death in all models. The High Lymphocyte Category is significantly associated with death in all but the stratified comprehensive risk factor model in which there was still a trend to significance. The Mid TWCC Category was strongly significant (p=0.01, Table 8.30) in the model adjusting for

comprehensive risk factors (CRF) but not significant with traditional risk factor (TRF) adjustment alone. Adjusting for risk factors had the opposite effect with the Mid Lymphocyte Category which was significant in the *a priori* model but only trended to significance when adjusting for traditional risk factors and was not significant when the model was adjusted for comprehensive risk factors. The Mid Neutrophil Category, the Mid Monocyte Category and the Mid and High Haemoglobin Categories were not significantly associated with death in any model.

Table 8.31: Combined cell types Cox proportional hazards analysis - death and all cell types by category.

Outcome - Death					
Cell Type		p Value	HR (95% CI)	Cox.zph	Global zph
Neutrophil Category	mid	0.20	1.49 (0.81-2.72)	0.85	**0.03**
	high	**<0.01 ****	**2.36 (0.13-4.24)**	0.71	
Lymphocyte Category	mid	**0.04 ***	**0.60 (0.37-0.98)**	**<0.01**	
	high	**<0.01 ****	**0.33 (0.20-0.57)**	0.38	
Monocyte Category	mid	0.58	1.16 (0.68-1.99)	0.85	
	high	0.08 #	1.68 (0.94-3.02)	0.16	
Haemoglobin Category	mid	0.24	0.75 (0.46-1.22)	0.48	
	high	0.14	0.69 (0.42-1.13)	0.48	

= p value = 0.1 to 0.05
* = p value = 0.01 to 0.05 (**bold** type)
** = p value <0.01(**bold** type)
HR = Hazard Ratio
NS = Not significant

A combined cell types Cox proportional hazards model was used to assess all cell subtypes and the outcome of death tested *a priori* and the results are displayed in Table 8.31. The significantly associated cell types were High Lymphocyte Category (<0.01, hazard ratio 0.33), High Neutrophil Category (<0.01, hazard ratio = 2.36), Mid Lymphocyte Category (<0.01, hazard ratio 0.60). The High Monocyte Category trended to significance but did not reach the 0.05 level (0.08, Hazard ratio 1.68, Table 8.31).

The probability of breach of the assumptions of the Cox model for all circulating cell types and major adverse event models was raised with Mid Lymphocyte Category significant at the 0.05

level (<0.01, Table 8.31) and the global zph for the combined model significant with 0.03. This raises suspicion that the Cox proportional hazards model is not appropriate to use for this data set and may generate invalid results.

This model was not adjusted for confounding variables as with previous models of only one cell type due to the eight degrees of freedom already required for this model. Population size and event incidence do not allow for meaningful adjustment with either traditional risk factors (TRF) or comprehensive risk factors (CRF) due to the number of degrees of freedom these models would require being beyond the power of this study. It was determined that further analysis of cell counts and risk factors was warranted and this is presented below using multi-model averaging.

8.6.8. Multi-model analysis for the outcome of death

For the outcome of death the traditional risk factors of entry age, gender, diabetes mellitus, hypertension, ischaemic heart disease, transient ischaemic attack and stoke as well as disease severity at presentation and the medication use of aspirin and statin were included in multi-model analysis with circulating cell counts Neutrophil Category, Lymphocyte Category, Monocyte Category, Haemoglobin Category and calculated Neutrophil/Lymphocyte Ratio (NLR) Category. Careful consideration of the evidence to support the variables was undertaken with Haemoglobin Category not significantly associated with mortality in any of the *a priori* Cox proportional hazards analysis and High Monocyte Category only trending to significance (Table 8.30). Previously published evidence for these associations was assessed[49,270-272,322] and both of these variables were included in the multi-model analysis because of the precedent associations in the scientific literature.[132] TWCC Category was not included in the multi-model analysis as it is an additive product of the neutrophil, lymphocyte and monocyte subtypes.

The statistical package R[354] was used to run all possible models using the explanatory variables above for the outcome of Death with the package MuMIn[355] (with K=15 parameters which are listed above). The models were then reduced to just the models that were not significantly different to the "best" single model using the $\Delta_i < 2$ function and are presented as the top model set (Table 8.32) with all the information required to interpret the analysis including degrees of freedom and log-likelihood ($\log(\mathcal{L})$) for each model.[368] Background information and equations are found in Chapter 4: Akaike Information criterion adjusted for sample size AICc (Equation 10); the Δi for each model (Equation 11); and Akaike weight w_i (Equation 13) summarised in Table 4.3.

Table 8.32: Multi-model analysis: top model set for death (Δ_i <2)

Component models:	df	log($\mathcal{L}$)	AIC_c	Δ_i	w_i
Age/ HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category / Smoking	11	-458.53	939.74	0.00	0.10
Age/ HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category	10	-459.76	940.09	0.35	0.08
Age/ HTN / Disease Severity / Lymphocyte Category / Neutrophil Category / Smoking	10	-459.87	940.31	0.57	0.07
Age/ HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category / Smoking / Statin	12	-457.76	940.33	0.59	0.07
DM/Age/ HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category / Smoking	12	-457.87	940.56	0.82	0.06
Age/ Gender / HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category	11	-459.14	940.97	1.23	0.05
Age/ Gender / HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category / Smoking	12	-458.12	941.04	1.30	0.05
Age/ HTN / Disease Severity / Lymphocyte Category / Neutrophil Category	9	-461.30	941.06	1.32	0.05
Age/ HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category / Statin	11	-459.21	941.11	1.37	0.05
Age/ HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / Neutrophil Category	12	-458.20	941.21	1.47	0.05
DM/ Age / HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category	11	-459.32	941.33	1.59	0.04
Age/ Gender / HTN / Disease Severity / Lymphocyte Category / Neutrophil Category / Smoking	11	-459.33	941.35	1.61	0.04
Age/Gender/HTN/IHD/Disease Severity / Lymphocyte Category / Neutrophil Category / Smoking / Statin	13	-457.23	941.41	1.67	0.04
Age/ Gender /HTN / Disease Severity/Lymphocyte Category / Neutrophil Category	10	-460.44	941.44	1.70	0.04
Age/ HTN / IHD / Disease Severity / Lymphocyte Category / Monocyte Category / Neutrophil Category / Smoking	13	-457.26	941.47	1.73	0.04
Age/ HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category / Smoking / TIA	12	-458.33	941.47	1.73	0.04
Age/ HTN / Disease Severity / Lymphocyte Category / Neutrophil Category / Smoking / Statin	11	-459.46	941.60	1.86	0.04
Age/ HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category / Smoking / Stroke	12	-458.42	941.66	1.91	0.04
Aspirin/Age/ HTN / IHD / Disease Severity / Lymphocyte Category / Neutrophil Category / Smoking	12	-458.46	941.74	2.00	0.04

AIC_c = Akaike information criterion corrected for sample size (Equation 10)
Δ_i = AICi-AICmin (Equation 11)
log($\mathcal{L}$) = Log likelihood of individual model ($\mathcal{L}$ from Equation 12)
w_i = Akaike weight (Equation 13)
IHD = Ischaemic Heart Disease
Disease Severity = Disease Severity at presentation
Smoking = Smoking Category (Never smoker, smoker – ex or current)
df = Degrees of Freedom
DM = Diabetes Mellitus
HTN = Hypertension

Of the top model set $\Delta_i < 2$, the simplest model had only nine degrees of freedom while two models had 13 degrees of freedom. The log($\mathcal{L}$) values range from -457.23 to -461.30

demonstrating comparable fit of the selected models. The individual AIC_c are not interpretable as they contain arbitrary constrains and are greatly affected by sample size[367] but once transformed to Δ_i with the best model given a Δ value of 0 and four other models having a Δ_i <1 the information loss in using those models over the "best" model is quantified.

The "best" model contained the variables Age, Hypertension, Ischemic Heart Disease, Disease Severity, Lymphocyte Category and Neutrophil Category and Smoking status (the first model presented in Table 8.32 with a Δ value of 0). The "best" model has been estimated to be the best but the w_i results show that no one model is supported as a being clearly superior over the other considered models with the "best" model having a probability of only 10% and the second model 8% and all other models from the top model set (with Δ_i <2) ≥ 4%. The w_i / w_j evidence support ratio demonstrates the empirical support for the "best" model is 2.5 times that of the nine models with w_i = 0.04 and twice that of the five models with w_i = 0.05.

The second best model for the outcome of death contained the same variables as the "best" model without smoking and results in a Δ_i of 0.36 which may be interpreted as having minimal information loss despite the removal of one degree of freedom. The w_i / w_j evidence support ratio confirms the empirical support for the "best" model is only 1.25 times that of the second best model.

This model selection uncertainty was incorporated by multi-model averaging which has the benefit of being able to include important information not included in the best model. This was done due to the w_i of the "best" model being <0.9 and to objectively account for uncertainty in the selection of the best model.[132,372] Multi-model averaging was then applied to make

inference from all of the models from the top model set with Δ_i <2 with adjustment for their supportive value and model probability. The variables from all the models in Table 8.32 were thus ranked according to the number of models (from the top model set with Δ_i <2) that each variable appeared in and factored for the complexity of each of those models using the Akaike weight for the model (w_i) to calculate the importance for each variable for the outcome of death. The results of ranking variables using this process are presented in Table 8.33. The subsequent averaged model is analysed to assess the coefficients and significance of contributing variables to the averaged model (Table 8.34).

Table 8.33: Relative variable importance from multi-model analysis for death

Variable	Importance	No. of models
Disease Severity at presentation	1.00	19
Neutrophil Category	1.00	19
Lymphocyte Category	1.00	19
Hypertension	1.00	19
Age	1.00	19
Ischaemic Heart Disease	0.75	14
Smoking	0.64	12
Gender	0.23	5
Statin	0.20	4
Diabetes Mellitus	0.11	2
Monocyte Category	0.09	2
Transient Ischaemic Attack	0.04	1
Stroke	0.04	1
Aspirin	0.04	1

Table 8.33 shows that five variables (Lymphocyte Category, Neutrophil Category, Entry Age, Hypertension and Disease severity at presentation) were present in all 19 of the models in the top model set Δ_i <2. The variables of Ischaemic Heart Disease and smoking were present in more than half the models in the top model set (14 and 12 respectively, Table 8.33). These variables collectively are the best predictors for the outcome of Death. The "best" model contained all of the best predicting variables. Gender, statin use, diabetes mellitus, Monocyte

Category, Transient Ischaemic attack, stroke and aspirin are inconsistent contributors to the Δ_i <2 top model set (Table 8.33).

Closer examination of the top model set (Δ_i <2, Table 8.32) in light of the results presented in Table 8.33 shows that five of the seven models from the top model set that do not containing smoking do contain IHD, and two of the four models that do not contain IHD contain smoking whilst the two variables appear together in nine of the total 19 models in the top model set.

The lower relative importance of gender is due to the fact it appeared in five models and also was not in any models with w_i >0.05. Gender is less likely to be a good predictor of death than the best predictors referred to earlier but may still have significant influence in the models in which it was included. Transient ischaemic attack, stroke and statin use were present in only one model each and are less likely to be strong predictors of death compared to the best predictors.

Neutrophil-Lymphocyte Ratio has the advantage of less degrees of freedom than including both the Neutrophil Category and Lymphocyte Category however was not in any of the top model set with Δ_i <2, although both Neutrophil Category and Lymphocyte Category appear together in all 19 models.

The model averaged coefficients were generated using the natural average method[132,372](Table 8.34) indicate the direction of effect for each variable with the standard error for each variable,

i.e. a negative coefficient indicates a negative effect on the outcome of death, meaning that death is less likely to occur.

Table 8.34: Full model averaged coefficients for Death

Variable	Standardised Coefficient	SE of coefficient	z value	Pr(>|z|)
Tissue Loss	1.58	0.76	2.08	0.04
Rest pain	1.15	0.78	1.48	0.14
High Neutrophil Category	1.03	0.29	3.60	<0.01
High Lymphocyte Category	-0.78	0.27	2.92	<0.01
Hypertension	-0.60	0.28	2.11	0.04
Mid Neutrophil Category	0.50	0.30	1.67	0.09
Intermittent claudication	0.39	0.73	0.53	0.59
Ischaemic Heart Disease	0.30	0.26	1.13	0.26
Mid Lymphocyte Category	-0.24	0.25	0.94	0.34
Smoking	0.14	0.15	0.91	0.36
Age	0.06	0.01	4.37	<0.01
Gender	-0.06	0.16	0.37	0.71
Statin	-0.05	0.14	0.36	0.72
High Monocyte Category	0.04	0.16	0.25	0.80
Diabetes Mellitus	-0.03	0.11	0.25	0.81
Transient Ischaemic Attack	-<0.01	0.08	0.11	0.92
Stroke	<0.01	0.07	0.08	0.93
Mid Monocyte Category	<0.01	0.09	0.06	0.96
Aspirin	<0.01	0.05	0.06	0.95

SE = Standard Error

Table 8.34 presents the multi-model inference results which combined the entire set of candidate models weighted by their support. The standardised coefficient and standard error were used to generate a z value and the probability of each z value is included in Table 8.34. Caution needs to be taken in interpreting these results from a null hypothesis testing paradigm that traditionally uses the probabilities of z statistics to accept or reject the null hypothesis.[132,368] These results are presented here to demonstrate the relative effect of coefficient and standard error on the individual variables in the averaged model and not in the binary significant, not significant way.[368] Importantly, variables with a non-significant p value are not discarded, because the overall model is better with them than without them. The Pr values are very useful

in the interpretation of the averaged model for variables that are likely to be correlated and hence partly substitutable in the averaged model. The low Pr values of High Lymphocyte Category, High Neutrophil Category and Age along with the presence of these variables in most or all of the top model set (Table 8.33) may be interpreted as these variables being key or non-substitutable in the averaged model for death. The comparatively larger Pr value of Smoking (0.36, Table 8.34), despite its presence in the "best model" and presence in more than half of the models in the top model set (Table 8.33) is an interesting finding. The larger Pr value of Smoking is indicative that when included in the same model as a correlated or substitutable variable it is expected that that both variables will have their level of significance reduced or eliminated compared to if only one of these variables was included. The averaged coefficients seen in Table 8.34 will always be weaker than the coefficients in the "best" model but the process is designed to be generous with inclusion over exclusion and will tend to underestimate the relative strength of variable effect compared with the "best" model.

The disease severity categories of Tissue Loss and Rest pain had the largest coefficients in the averaged model (1.58, 1.15 respectively, Table 8.34) although they also had the largest standard errors for these coefficients (0.76, 0.78 respectively, Table 8.34). The z value of 2.08 for variable Tissue Loss and the subsequent Pr(>|z|) 0.04 are included to quantify the strength of this effect which is greater than that of Rest Pain which demonstrated a smaller coefficient with a similar size standard error which resulted in a z value of 1.48 and larger Pr(>|z|) of 0.14 meaning we can be less confident of the magnitude of this effect. The disease severity category of Intermittent Claudication was also positively associated with death with a larger effect than all traditional risk factors except hypertension. The variable has a standard error greater than

the coefficient of the variable which is why the z value is so much less (0.53, Table 8.34) than the disease severity categories of Tissue Loss and Rest Pain.

Both High Neutrophil Category and Mid Neutrophil Category exhibit strong influence in the averaged model for the outcome of death with both exhibiting larger positive coefficients than any of the traditional risk factors. The High Neutrophil category has a positive coefficient that is twice that of the Mid Neutrophil category with similar standard error for both groups, generating the largest z value of the categorical variables of 3.6 ($Pr<0.01$, Table 8.34).

High Lymphocyte Category displayed the strongest negative association with Death (-0.78, Table 8.34) indicating that a High Lymphocyte Category was protective for the outcome of Death. The High Lymphocyte Category demonstrated a strong effect with comparatively small standard error (0.27, Table 8.34) generating a large z value of 2.92($Pr<0.01$, Table 8.34). The Mid Lymphocyte Category a more modest negative coefficient (-0.24, Table 8.34) with a larger standard error than coefficient the strength of this association is less certain.

Hypertension had the largest effect of all the traditional risk factors and also had a negative coefficient (-0.60, Table 8.34) suggesting a significant negative association with Death over the course of this study. Ischaemic heart disease despite appearing in 14 of the 19 models with $\Delta_i < 2$, had a more modest effect with coefficient of 0.30 and standard error of 0.26 (Table 8.34). Smoking was the other traditional risk factor to appear in more than half of the models with $\Delta_i < 2$ with a smaller effect in the averaged model.

Age is the only continuous variable in this multi-model analysis and although the coefficient is seeming more modest than other variables (0.06, Table 8.34) it has the smallest standard error (0.01) and as such generated the largest z value (4.37, <0.01 Table 8.34). The coefficient for age applies to the value for that continuous variable and it has a strong effect on the outcome, in this case death. For example in an 80 year old patient the age term would be a function of the coefficient (0.06, Table 8.34) and the value 80.

The remaining traditional risk factors of Aspirin and Statin use, Diabetes Mellitus, Transient Ischaemic Attack and Stroke along with both Mid and High Monocyte Category exhibited modest effects that should be interpreted with caution. It is possible that these variables interact with one or more of the other variables in the model and the better predictor may suffice but in the interests of caution these variables have been included in the average model because it is not possible to tell from this data set alone which of these variables can be safely excluded. Care should be exercised in interpreting the direction of association of these weakly correlated variables as assigning the incorrect sign to a weak parameter is a possibility in any inference analysis.[384,385]

8.7. Discussion for death

The aim of this study was to combine the assessment of all the circulating cell types for the endpoint of death and subsequently generate a model that combines the circulating cell counts with established risk factors and clinical severity of disease. This is the first study to use multi-model averaging from the Information-Theoretical paradigm for model creation for the outcome of death in patients with peripheral arterial occlusive disease. The resultant model from this study may be used as a risk prediction tool for death in peripheral arterial occlusive disease patients that may be applied by the clinician at the bedside. The development of a model for death in this population not only explores the true relationships and relative predictive value of the circulating cells for mortality but has the potential to be applied to increase patient compliance with lifestyle modifications and medical treatment,[133] better guide risk stratification models for patient treatment[137] and also guide further research into understanding of the pathogenesis of death in this population.

Disease severity and the circulating neutrophil and lymphocyte cells were stronger predictors of death using multi-model inference than the traditional atherosclerotic risk factors of gender, hypertension, ischaemic heart disease, stroke, transient ischaemic attack, smoking and diabetes mellitus. Disease Severity, Lymphocyte Category and Neutrophil Category along with age, and hypertension were present in all 19 of the top model set for death Δ_i <2 (Table 8.33). The traditional risk factors of ischaemic heart disease and smoking were present in more than half of the top model set; while gender, statin, diabetes mellitus and Monocyte Category were inconsistent contributors. Multi-model analysis was used for the analysis of this complex system because controlling for the enormous number of factors affecting outcome is impractical,[132,386] and many of the variables may exhibit small but clinically important

effects.[302] Only variables with strong biological reasoning and established *a priori* relationship were included in the multi-modelling analysis[132] and this evidence was established in the null-hypothesis testing section of this chapter prior to commencing multi-model analysis. The TWCC was not included in the multi-model analysis because it is a function of the addition of other cell types.

Disease severity at presentation was present in all 19 of the top model set with Δ_i <2 (Table 8.32) and the two strongest variable effects in the averaged model for death were Tissue loss (1.58 ±0.76) and Rest Pain (1.15 ±0.78, Table 8.34). The effect of Tissue Loss for the outcome of death was 2.6 times that of hypertension and 5.2 times that of ischaemic heart disease. Tissue Loss exerted 4 times the effect of the other Disease Severity category Intermittent Claudication on the outcome of death. The effect size of Tissue Loss was large enough to generate a z value of 2.08 despite the large standard error of 0.76 (Pr=0.04, Table 8.34). The objective and quantified finding that Disease severity is the most important predictor of mortality in patients with peripheral arterial occlusive disease has important implications for future research in this field as previous literature has inconsistently reported and adjusted for clinical severity of disease when investigating this patient group.[49,122,222,320] The strength of the variables Tissue Loss and Rest Pain should be interpreted in light of their generation not only by the quantified process of model selection with model likelihoods and estimates of model precision, but this accuracy of parameter estimates for the components of the averaged model is not achievable through null hypothesis testing statistical methods.

This research methodology demonstrates Disease Severity and the High Neutrophil Category to be strong predictors for the outcome of death. A causative relationship between these

variables has not been established and is not necessary to be established for the model to function as it is. However, to progress understanding of the underlying disease process it is important to consider whether the tissue ischaemia of more severe peripheral arterial disease causes activation of the neutrophils, increasing their adhesion and reducing their deformability and thus causing adverse effects in distant organs,[230,231,341] or the high count of circulating neutrophils are causative of the lower limb tissue ischaemia through plaque disruption[397] as a marker of atherosclerotic disease burden. A longitudinal study design incorporating cell functional assessment would be one method of better defining this relationship.

Neutrophil Category and Lymphocyte Category were the only cell types present in all 19 of the top model set Δ_i <2 (Table 8.32) along with Disease severity, Hypertension and Age. The calculated Neutrophil-Lymphocyte Ratio did not feature in any of the 19 models within the top model set for the outcome of death although neutrophil-lymphocyte ratio has the advantage of less degrees of freedom than including both the Neutrophil Category and Lymphocyte Category. This is consistent with the findings of multi-model analysis for the outcome of major adverse event in this population. The quantitative comparison of variable contribution to models for outcome has been previously unavailable with null hypothesis testing based statistics and this new paradigm of Information-Theoretical statistical analysis should be employed to more accurately direct future research.

High Neutrophil Category had the largest effect size in the averaged model an effect twice that of the Mid Neutrophil Category with a similar size standard error (Table 8.34). Both the High Neutrophil Category and High Lymphocyte Category exhibited a larger effect than that of the best traditional risk factor Hypertension. The High Lymphocyte Category had the largest

negative effect in the averaged model with coefficient -0.78 (<0.01, Table 8.34), 3.25 times that of the Mid Lymphocyte Category with a coefficient of -0.24 (z score 0.94, Table 8.34), both of which had a larger effect than the traditional risk factors of smoking, diabetes, Transient Ischaemic Attack, Stroke, Aspirin and Statin use. This re-enforces the early findings of Dormandy and Murray[24] who reported the absence of predictive value for the traditional risk factors of hypertension, diabetes, smoking and high plasma cholesterol levels and suggested that these risk factors for the development and early progression of atherosclerosis are not necessarily prognostic factors in the final stages of the disease.[24]

Both High and Mid Monocyte Categories feature in the averaged model although Monocyte Category was present in only two of the 19 best fit models (Δ_i <2) both of which had $\Delta_i \geq 1.47$ with w_i of 0.05 and 0.04. Because of this low Akaike weight weaker effects in the averaged model for the outcome of death are expected. Further research with a larger sample may clarify this relationship, but currently the High and Mid Monocyte Categories contribute weakly to the averaged model for death.

The strongest effect from a traditional risk factor in the averaged model for death was the negative coefficient of hypertension of -0.60±0.28 (<0.04, Table 8.34) which was consistent with the negative coefficient for hypertension in the averaged model for major adverse event. The reason for this negative association is not clear from the analysed data with hypertension traditionally having a positive association with mortality.[100,398] As discussed with regard to the averaged model for major adverse event; treatment of hypertension in peripheral arterial occlusive disease has been demonstrated to lower the incidence of mortality in population studies.[104,399,400] The influence of antihypertensive medications at recruitment, any changes in

anti-hypertensive medication, or correlation with non-invasive blood pressure measurements were outside the scope of the current study and warrant further investigation in this population preferably with consideration of the Information-Theoretical approach.

Smoking, despite being present in 12 of the 19 top model set with Δ_i <2 and generating an importance of 0.64, displayed a relatively small effect (0.14±.015) having approximately 9% of the predictive power of the disease severity group Tissue Loss and 13.6% the predictive power of High Neutrophil Category. One explanation for this is the established relationship between circulating cell counts especially neutrophils and number of cigarettes smoked,[383] and the effect of cigarette smoking is better accounted for in the outcome model by the circulating cell counts that the patient report of cigarette smoking. Smoking should still be included in the averaged model for the outcome of death despite the lower predictive power as the effect of this variable is not replaced by the circulating cell counts alone.

This study convincingly demonstrates the importance of adjusting for confounding factors when undertaking null hypothesis testing (Cox proportional hazards used in this study) of circulating cell types with the outcomes of death in a population of patients with peripheral arterial occlusive disease. Variable incorporation into multi-model analysis of outcomes may be appropriate on the basis of *a priori* investigations and established literature alone[132] but the adjustment for traditional risk factors and comprehensive risk factors was performed to enable comparison of the investigated population with previously published populations of patients with peripheral arterial occlusive disease. Inconsistency of adjustment for confounding factors in the published literature examining this population has been described[223] and in this study is

demonstrated to have a significant effect on the observed significance of associations with death.

High TWCC was associated with the endpoint of death in all Cox proportional hazards models although variations in the variables adjusted for in published studies makes direct comparison problematic. In the stratified model that adjusted for comprehensive risk factors High TWCC had a hazard ratio of close to 2 (Table 8.19), which was less than the hazard ratio found by Arain et al.[222] who found a hazard ratio of 4.09 (1.57-10.64) when they adjusted the Cox proportional hazards model for age, sex, smoking, hypertension, ischaemic heart disease/stroke, serum creatinine and ankle brachial index. The hazard ratio for death found in this study is greater than the relative risk of vascular death (1.51, 1.20–1.89) of the patients in the upper TWCC quartile described by Grau et al.[69] in the CAPRIE study which included patients with stroke and ischaemic heart disease as well as peripheral vascular disease. Haim et al.[404] described a similar relative risk to Grau et al.[69] in patients with coronary heart disease (1.47)[404] although the relative risk does not take into account the timing of deaths.[405] In the study of 108 patients with critical limb ischaemia by Pedrinelli[322] elevated TWCC had a risk ratio for death of 3 (2.76-3.28) on univariate analysis which was not persistently significant on multivariate analysis. The findings of TWCC association with mortality were not replicated in the study of 1021 patients undergoing major vascular surgery by Bhutta et al.[49] who did not demonstrate a significant association of TWCC with two year mortality (univariate odds ratio 1.02 (0.95-1.09.) and Haumer et al.[72] who also did not demonstrate a significant association of TWCC with death over a median follow up of 20 months. This may be due to methodological differences between the studies with 51.8% of the population studied Bhutta et al.[49] undergoing

aneurysm repair, a population that was excluded from the current study and this study having a median follow up four times longer than the study by Haumer et al.[72]

The importance of adjusting for disease severity was seen in the Cox proportional hazard results of Mid TWCC Category testing with death where the 0.05 level of significance was only reached in the comprehensive risk factor adjusted Cox models (Table 8.19 and Table 8.30) raising the possibility that one of the confounding factors not adjusted for in the traditional risk factor adjustment may have masked the effect of TWCC on death. It is likely that this was disease severity, previously established as an important confounding variable to consider with Tissue Loss significant (p = 0.02) when included in the comprehensive risk factor adjusted model. Once the Cox model for TWCC and death was adjusted for the comprehensive risk factors and stratified for the other significant categorical variables (ischaemic heart disease and Tissue Loss) Mid TWCC was demonstrated to be significant (p = 0.02) with a hazard ratio of 2.00 (1.12-3.55) greater than that of the High TWCC Category. TWCC was not included in the multi-model analysis because it is a direct function of the addition of cell subtypes but future research should compare TWCC with cell subtypes as predictors of death through multi-model analysis with different design.

The High Neutrophil Category was significantly associated with death in all Cox models at the <0.01 level establishing precedent for its inclusion into multi-model analysis. Hazard ratio for the High Neutrophil Category in the comprehensive risk factor adjusted model was 2.49 (1.43-4.32) with age the only other significant variable (<0.01, Table 8.21). The High Neutrophil Category was also highly significant in the combined cell types Cox model for death (<0.01, Table 8.31) with a similar hazard ratio of 2.36 (0.13-4.24). This is lower than the hazard ratio

of 3.4 (1.34-8.53)[72] found by Haumer et al.[72] after adjusting for comprehensive risk factors who followed 398 patients with symptomatic peripheral arterial occlusive disease for a median of 20 months. The hazard ratio in this study of High Neutrophil Category for death was greater than reported by Grau et al.[69] in the CAPRIE study 1.86 (1.47–2.36) although specific comment was made that they may have underestimated risk by 30-35% for neutrophil count due to regression dilution.

High Lymphocyte Category was significantly negatively associated with the endpoint of death in all Cox proportional hazards models except the comprehensive risk factor adjusted model stratified for other significant variables. In the comprehensive risk factor adjusted Cox proportional hazards model for death the High Lymphocyte Category was statistically significant (0.02, hazard ratio 0.54, Table 8.23) with the other statistically significant variables smoking, ischaemic heart disease, Tissue Loss and Hypertension. Once this model was stratified for other significantly associated categorical variables the High Lymphocyte Category only trended toward significance (0.10, Table 8.23) It is not clear if this effect is related the potential breach of the proportionality assumptions of the Cox model by the Mid Lymphocyte Category or the relationship of one of the other statistically significant variables. The Mid Lymphocyte Category was significantly associated with the outcome of death in the *a priori* model only (0.04, Table 8.23) with hazard ratio of 0.61 (0.38-0.99, Table 8.23) indicating that the Mid Lymphocyte Category was negatively associated or protective for the outcome of death. The Mid Lymphocyte Category had a significant Cox.zph score in all models except the stratified model for comprehensive risk factors indicating a breach of the assumptions of proportionality of the Cox models. The Mid Lymphocyte Category was also statistically significant in association with death in the combined cell types Cox model with

hazard ratio 0.60 (0.37-0.98) although the assumptions of proportionality were breached in this model. Tests of proportionality have not been published commonly in studies assessing outcome in patients with peripheral arterial occlusive disease, and as the aim is to investigate and describe a complex biological system the most appropriate analysis needs to be again considered with multi-model analysis considered superior for analysis of complex models with proven interactions between variables with small effects of some or all of the variables.[302] While the protective effect of relative lymphocytosis has been observed in other large studies[69,205,208] the underlying pathophysiological mechanisms of benefit from these lymphoid derived cells, possibly from a more competent immune response, requires further research.

High NLR Category was significantly associated with death in all Cox models. The High NLR Category was associated with death at the <0.01 level in all models except the stratified comprehensive risk factor model (0.04, Table 8.29) which stratified for the other significantly associated comprehensive risk factors (age, ischaemic heart disease, disease severity – Tissue Loss and smoking). In this stratified model adjusting for comprehensive risk factors the hazard ratio of 1.84 (1.02-3.31, Table 8.29) was lower than that of the other models suggesting that some of the hazard for association with death that was attributed to the High NLR Category in the model adjusted for traditional risk factors with stratified ischaemic heart disease (hazard ratio 2.25, Table 8.29) may have been due to one or more of the confounding variables in the comprehensive risk factor group. This is a similar to the results reported by Spark et al.[260] who reported a hazard ratio of 2.3 (1.2-4.2) using a Cox stepwise regression model adjusted for age, stroke, previous heart attack, statin use, renal failure, smoking history and elevated troponin in a cohort of 151 patients with critical limb ischaemia, and Bhutta et al.[49] who reported a multivariate odds ratio 2.21 (1.22-4.01) with NLR >5 in 1021 patients undergoing vascular

surgery. Importantly, the calculated neutrophil-lymphocyte ratio did not feature in any of the 19 top models from multi-model analysis with Δ_i <2 (Table 8.32) for the outcome of death, similar to the findings of multi-model analysis for the endpoint of major adverse event. This study demonstrates objectively in two separate multi-model analyses that despite NLR being significant in null-hypothesis testing when combined with Neutrophil Category and Lymphocyte Category in multi-model analysis the information lost using the NLR for the outcome of death is greater than the statistical advantage that should be seen from less degrees of freedom. To clarify, this finding is not disproving that NLR is associated with death or major adverse event, NLR is just inferior to the inclusion of both Neutrophil Category and Lymphocyte Category when creating models for the outcome of death and major adverse event in this population. This has important implications in the interpretation of previous research and demonstrates an inherent problem with the null-hypothesis testing paradigm as stepwise regression is unable to compare models without model likelihoods or estimates of precision,[306] making this relationship difficult to elucidate with previously applied methods. As a result of the findings of this study inclusion of both Neutrophil Category and Lymphocyte Category should be considered superior to the inclusion of Neutrophil-Lymphocyte Ratio in future models for the outcome of death in patients with peripheral arterial occlusive disease.

High Monocyte Category was not significantly associated with death in any of the Cox models although did trend to significance (0.06, Table 8.25). The hazard ratio of the High Monocyte Category of 1.69 (0.98-2.84) in the comprehensive risk factor adjusted model was greater than that in the CAPRIE study[69] where the upper quartile monocyte count vascular death Risk Ratio 1.29 (1.06–1.55) although by self-report they may have underestimated risk by up to 50% for monocyte counts due to regression dilution. High Monocyte Category displayed a similar trend

to significance in the combined cell types Cox model (0.08, Table 8.31) with hazard ratio 1.68 (0.94-3.02), the Mid Monocyte Category was not significantly associated with the outcome of death in any Cox Models. While monocyte derived macrophages have been described as mediating all the stages of atherosclerotic plaque formation and rupture,[47] and patients with peripheral arterial occlusive disease having monocytes that are more active than control groups,[265] the pathophysiological mechanisms by which monocytes lead to increased risk of death in this population require further investigation.

Neither Mid nor High Haemoglobin Category demonstrated a significant association with death in any of the Cox proportional hazards model. A finding that is consistent with the Dormandy and Murray[24] who also did not demonstrate significant association of haemoglobin with mortality in patients with Intermittent Claudication. Haemoglobin has previously been associated with two year mortality in patients undergoing major vascular surgery with univariate analysis of Haemoglobin (g/dL) demonstrating an odds ratio of 0.81 (0.72-0.91) but this association was not significant when adjusted for other risk factors.[49]

With these complex interactions established through null hypothesis testing analysis between circulating cells as markers of systemic inflammation, risk factors for vascular disease and clinical disease severity; multi-model analysis and averaging was undertaken. Multi-model analysis allowed comparison of all the variables and their association with the endpoint of death and may be used to infer the key underlying biological processes at work.[132,386] The multi-model analysis confirmed most of the highly significant findings from the Cox proportional hazards analysis with Neutrophil Category and Lymphocyte Category featuring in all 19 of the top model set with Δ_i <2. High Neutrophil-Lymphocyte Ratio was significant

in all Cox Proportional Hazards analysis but did not feature at all in the top model set for the outcome of death despite having the significant mathematical advantage of less degrees of freedom to include Neutrophil-Lymphocyte Ratio instead of Neutrophil Category and Lymphocyte Category. This is clinically important as quantitatively comparing which of these measures are best to include in a model for the outcome of death is problematic using null hypothesis testing statistical analysis. Monocyte Category which trended to significance in the Cox Proportional Hazards analysis was only in two of the top model set and produced a relatively weak positive coefficient in the averaged model although was still included as an important variable. This effect of Monocyte Category on the model for death in patients with peripheral arterial occlusive disease may be clearer from replication in a larger study population.

As described in Section 8.4 the developed averaged model may be applied by the clinician at the bedside using a simple computational application for smartphone. From the results of this multi-model inference analysis, Lymphocyte category and Neutrophil Category should be combined with clinical disease severity, patient age, history of hypertension and ischaemic heart disease in the model to predict death in patients with peripheral arterial occlusive disease. Monocyte Category, Smoking status, history of Transient Ischaemic Attack or Stroke, Diabetes Mellitus and Statin use would best have their role in the model clarified by replication with another population, however from this study alone they add meaningfully to the model and should be included. This proposed handheld application to assist the clinician to predict and communicate risk of death may help select the most appropriate treatment for individual patients[137] and may communicate risk to the patient in a way that enhances adherence to

lifestyle changes and medication use[133] that have been shown to improve outcomes in patients with peripheral arterial occlusive disease.[83,85,135,136]

As suggested for the outcome of major adverse event, the predictive value of the developed model for death in patients with symptomatic peripheral arterial occlusive disease would be confirmed and refined by replication of this study method in a larger population. Multi-model analysis to allow direct comparison of TWCC with subtypes of lymphocyte, neutrophil and monocyte for the outcome of death should be considered. As suggested for the outcome of major adverse event, the protective effect of hypertension or the treatment applied for hypertension plus the Ankle Brachial Index as an objective measure of the severity of peripheral arterial occlusive disease deserves further investigation. Expansion of the study population to include people with asymptomatic peripheral arterial occlusive disease would aid in identifying asymptomatic people at the highest risk of death who would benefit most from lifestyle changes and medical management. Models for mortality developed using multi-model analysis will be better able to identify underlying biological processes and should be used to guide future pathophysiological and therapeutic research into peripheral arterial occlusive disease.

8.8. Conclusions for death

Multi-model averaging is a relatively new statistical paradigm and is the preferred statistical method for analysing data from complex biological systems needing to account for multiple co-variables of modest effect that interact to produce clinically relevant outcomes and is here used to analyse death for patients with peripheral arterial occlusive disease. Multi-model averaging in this study has demonstrated that circulating cell counts of lymphocytes and

neutrophils together with disease severity at presentation, hypertension and age are more important in models to predict the outcome of major adverse event than other traditional risk factors.

Tissue Loss and Rest Pain were the two strongest predictors of death in the best averaged model in which the effect of the disease severity category Tissue Loss was 2.6 times that of hypertension and 5.2 times that of ischaemic heart disease. Intermittent Claudication also had a larger effect than the traditional risk factors of ischaemic heart disease, smoking, Diabetes mellitus, Transient Ischemic Attack, Stroke, Aspirin and Statin use. Disease severity should continue to be reported and accounted for when examining outcomes in patients with peripheral arterial occlusive disease.

Analysis using Information-Theoretical framework has shown that the inclusion of Lymphocyte Category and Neutrophil Category together provide better modelling for the outcome of death than the combined Neutrophil-Lymphocyte ratio. Lymphocyte Category and Neutrophil Category were in all of the models in the best model set.

The protective role of lymphocytes and hypertension (or the subsequent management) in the averaged outcome model for death in patients with symptomatic peripheral arterial occlusive disease deserves further research. Initially in the form of replication of these results in other populations of patients with peripheral arterial occlusive disease preferably with the power to investigate hypertensive management. Future expansion into pathophysiological research would then be able to determine if these protective effects may be exploited clinically.

Multi-model inferencing should be applied to larger data sets of patients with peripheral arterial occlusive disease to provide valuable information about the association of cell counts with outcomes in this population. Once the nature of these associations is refined not only will more accurate models be generated to predict the likelihood of death for individual patients and guide treatment selection, aid patient compliance with lifestyle change and medical therapy but future pathophysiological research and new treatment options will be able to be generated.

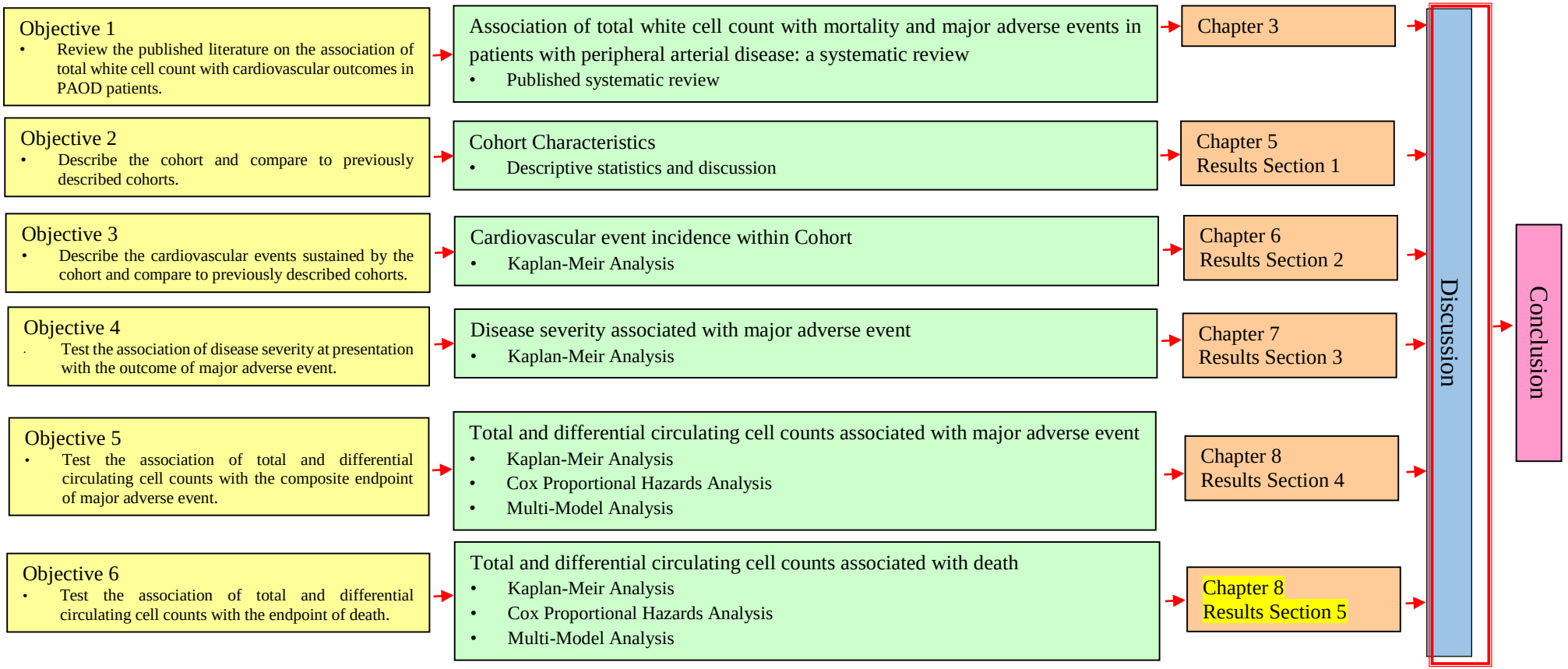

Figure 9.1: Schematic overview of thesis with red box highlighting current position within document - Discussion

9. Discussion

This is the first study to use multi-model averaging to assess the contribution of circulating cells, severity of disease and traditional risk factors with cardiovascular endpoints in patients with peripheral arterial occlusive disease. This new paradigm of hypothesis testing using the Information-Theoretic approach of multi-model averaging is more appropriate and meaningful than traditional null hypothesis testing approaches for patients with peripheral arterial occlusive disease. Outcome model development has important implications for the substantial population of patients with peripheral arterial occlusive disease estimated to 202 million adults worldwide.[13] Multi-model averaging has demonstrated that disease severity and the circulating cells of inflammation (neutrophils, lymphocytes and monocytes) are better predictors of major adverse event and death than the risk factors associated with the development of peripheral arterial occlusive disease. Through the process of multi-model averaging these results have been combined to produce outcome models for major adverse event and death in patients with peripheral arterial occlusive disease.

Limitations of the traditional null hypothesis testing approach have been demonstrated by the complex interplay of risk factors, circulating cells of inflammation and clinical disease severity. Adjustments in null hypothesis testing methods have significant effects on the relationships of variables such as circulating cells and outcomes. The variables adjusted for in published literature involving patients with peripheral arterial occlusive disease are inconsistent, with one potential reason that publication bias leads to selection of statistically significant results for publication.[406,407] This consequence is based on the inherent misunderstanding that null hypothesis testing is fundamental to the scientific method and can lead to a false sense of objectivity and rigor in statistical analysis and interpretation of research data.[408]

Null hypothesis testing methods traditionally utilise some form of step-wise regression to generate models with multiple predictors. Irrespective of the method used[305] this process completely ignores model selection uncertainty[358] and results in significant bias in parameter estimation.[132,358] Subsequently inappropriate focus is placed on the single "best" model, the consequences of which are not only erroneous conclusions,[358] but potentially misdirection of future research. These problems may be more pronounced when the predictors are correlated[409] or contain equivocal variables close to statistical significance[132] as they have been demonstrated to be in this population of patients with peripheral arterial occlusive disease. While these limitations of the null hypothesis testing method are being increasing recognised in other scientific disciplines[358,360,362,368,410] they remain rarely commented on in medical research. Traditional null-hypothesis testing still has a place in scientific research, it is just comparatively less informative compared with modern methods of analysis.[362] The Information-Theoretic approach arises strongly from the science of likelihood theory[132] and objectively compares models created to approximate an unknown reality[367] through measured observations. The Information-Theoretic approach is good with multiple predictors[410] and is able to account for model selection uncertainty and provide scientific results based on quantified support from collected data.[411] In other scientific fields initial uptake of this new paradigm was slow, potentially due to unawareness of this statistical technique, and also that this newer philosophy is a significant departure from traditional statistical training.[412]

The best fit averaged model calculated in this study for both the outcome of major adverse event (Table 8.17) and death (Table 8.34) in patients with peripheral arterial occlusive disease may be used to weight the predictive value of disease severity, circulating cells and clinical

risk factors to generate outcome predictions for individual patients with peripheral arterial occlusive disease. The consistency of variables in the top model set for both major adverse event and death is presented in Table 9.1 with the standardised coefficients for both averaged models presented in Table 9.2. The consistency of clinical disease severity and age which were present in all of the top model set in both averaged models for death and major adverse event can be seen in Table 9.1 with strong coefficients of Rest Pain and Tissue Loss in both averaged models (Table 9.2) is an important message for future research which should include and adjust for these variables in future investigations. Correlation of clinical disease severity with the objective measure of Ankle Brachial Index is advised and comparison using multi-model analysis would assess which of these measures of disease severity is best to include in models for these outcomes.

Table 9.1: Relative variable importance in multi-model analysis for major adverse event and death

Variable	**Major Adverse Event**		**Death**	
	Importance	**No. of models (out of 18)**	**Importance**	**No. of models (out of 19)**
Age	1.00	18	1.00	19
Disease Severity	1.00	18	1.00	19
Gender (Stratified in MAE analysis)	1.00	18	0.23	5
Hypertension	0.96	17	1.00	19
Ischaemic Heart Disease	0.92	16	0.75	14
Lymphocyte Category	0.90	16	1.00	19
Monocyte Category	0.73	13	0.09	2
Neutrophil Category	0.59	11	1.00	19
Smoking	0.27	5	0.64	12
Transient Ischaemic Attack	0.16	3	0.04	1
Statin	0.09	2	0.20	4
Diabetes Mellitus	0.09	2	0.11	2
Stroke	Not significant		0.04	1
Aspirin	Not significant		0.04	1

MAE = Major Adverse Event

Table 9.2: Full averaged model coefficients for major adverse event and death

Variable	Major adverse event		Death	
	Standardised Coefficient	SE of coefficient	Standardised Coefficient	SE of coefficient
Rest pain	0.71	0.59	1.15	0.78
High Monocyte Category	0.56	0.43	0.04	0.16
High Lymphocyte Category	-0.53	0.30	-0.78	0.27
Hypertension	-0.53	0.28	-0.60	0.28
Mid Lymphocyte Category	-0.49	0.28	-0.24	0.25
Tissue Loss	0.41	0.58	1.58	0.76
High Neutrophil Category	0.39	0.39	1.03	0.29
Intermittent claudication	-0.39	0.54	0.39	0.73
Ischaemic Heart Disease	0.38	0.23	0.30	0.26
Mid Monocyte Category	0.23	0.27	<0.01	0.09
Mid Neutrophil Category	0.18	0.26	0.50	0.30
Age	0.06	0.01	0.06	0.01
Transient Ischaemic Attack	-0.05	0.17	-<0.01	0.08
Smoking	0.04	0.09	0.14	0.15
Statin	-0.01	0.07	-0.05	0.14
Diabetes Mellitus	-0.01	0.07	-0.03	0.11
Gender	Stratified in model		-0.06	0.16
Stroke	Not significant		<0.01	0.07
Aspirin	Not significant		<0.01	0.05

SE = Standard Error

Monocyte Category was the most important circulating cell in the averaged model for major adverse event (Table 9.2) while it was less important in the model for death raising the possibility that Monocyte Category may be a better predictor of the outcomes of heart attack or stroke than the outcome of death alone in the population of patients with peripheral arterial occlusive disease. Monocytes play a key role in the formation of atherosclerotic plaque[46] and their accumulation in atherosclerotic plaque has been described preceding plaque rupture[413] but why the circulating monocyte count may be a better predictor of heart attack or stroke rather than death remains unclear and warrants further investigation. Circulating monocyte count has also been reported to decrease following revascularisation for critical limb ischaemia[267] and the effects of this in a large population would be worthwhile to assess if the observed reduction in circulating monocytes after revascularisation results in reduced risk of major adverse event. This study was not significantly powered to investigate the effects of revascularisation or the

endpoints of heart attack or stroke individually, but replication of this study in a larger population would enable that analysis and may give important insight into the role of circulating monocytes and their role in predicting individual cardiovascular adverse events.

Neutrophil Category was the strongest circulating cell count predictive of death appearing in all of the top model set for death (Table 9.1) with the largest coefficient of the circulating cells (Table 9.2), but was less well represented in the top model set for major adverse event appearing in only 11 of the 18 top models whilst still having a stronger positive coefficient for major adverse event than any of the traditional risk factors. This observed difference that Neutrophil Category is potentially less predictive of heart attack and stroke than death alone is not explained by this study. One potential reason may be methodological, with a heart attack that resulted in death recorded as death alone and Neutrophils having strong positive association with mortality following heart attack.[205,234,235] Neutrophilia has been associated with the development of coronary artery disease,[176] and experimentally neutrophils have been demonstrated to have a proinflammatory role in atherosclerosis.[414] It is not clear from the design of this study the mechanism by which the high circulating neutrophil count contributes to the risk of death. Neutrophils have previously been demonstrated to be involved in the development of ischaemic events through adherence to the endothelium, and plaque disruption through the release of superoxide radicals, proteolytic enzymes and arachidonic acid metabolites,[190,227,228] reduced deformability[232] or the aggregation with platelets that contributes to microvascular and macrovascular plugging promoting infarction,[72,228,229] leading to organ compromise and subsequent death. That this study demonstrates

Lymphocytes were consistent negative predictors in the averaged model for both major adverse event and death (Table 9.1, Table 9.2). T lymphocytes have long been established to have a role in plaque establishment, secreting cytokines and growth factors that control migration and proliferation of smooth muscles cells into plaque, and play a role in the production of procoagulant tissue factor that triggers thrombus formation following plaque rupture.[46] However despite the protective effect of relative circulating lymphocytosis being observed in large studies,[69,205,208] and the known atheroprotective effects of regulatory T lymphocytes and B lymphocytes[415] the link between circulating lymphocytosis and the pathophysiology of this atheroprotection remains unclear. One possibility is that a more competent immune response indicated by the high lymphocyte count restricts the progression of atherosclerotic disease but would require further research. Given the strength and consistency of the protective association of circulating lymphocytes with the outcomes of major adverse event and death in this study this is an area that warrants further investigation. Lymphocyte subset analysis may inform relative contributions of lymphocyte types to this observation.

Neutrophil-Lymphocyte Ratio did not feature in either of the top model sets using the multi-modal approach (Table 9.1). Although a strong relationship with both death and major adverse event was established through null-hypothesis testing analysis this was not as important in the modelling for either outcome as the inclusion of both Neutrophil Category and Lymphocyte Category. This is the first study to be able to objectively compare the relative contributions of variables to outcome in this population through the use of multi-model analysis; a result not achievable using traditional null hypothesis statistical approaches. This finding demonstrates that while Neutrophil-Lymphocyte Ratio is associated with death and major adverse event it is a less powerful predictor in outcome models than inclusion of both cell subtype categories.

Hypertension was also shown to have a strong and consistent effect as a negative predictor of both major adverse event and death in this population and was an unexpected finding, as hypertension traditionally has a positive effect on mortality.[100,398] Hypertension was present in 17/18 of the top model set for major adverse event and all of the top model set for death (Table 9.1). Hypertension demonstrated a consistent negative standard co-efficient for both major adverse event and death greater than -0.50 (Table 9.2). Treating hypertension has shown mortality benefit in peripheral arterial occlusive disease patients[400] and other patient groups,[416-418] although treatment of hypertension is less intensive in the peripheral arterial occlusive disease population than in patients with coronary artery disease.[5] This study was not designed to examine the relative contribution of different antihypertensive agents to the reduction in risk of major adverse event or death but is however an important finding. Multi-model inference would be an ideal tool to use for this analysis in a population of patients with peripheral arterial occlusive disease where controlling for all other factors affecting outcome including their interactions would be impossible.

Other traditional risk factors of smoking, transient ischaemic attack, stroke, diabetes mellitus, aspirin and statin use contributed inconsistently to the models in the top model set for both major adverse event and death (Table 9.1). Small coefficients with relatively large standard error were observed for these variables in the averaged model (Table 9.2), and the direction of prediction is not clear with most values close to 0. From this study alone these variables contribute significantly to the averaged model and should be included but this role would be clarified by replication of this study in a larger population. As previously discussed these risk factors may not be as important predictors of outcome in symptomatic peripheral arterial

occlusive disease as they are in the establishment of the disease.[24] Another explanation is that the circulating cell counts better represent the *contribution* of the risk factor/s to the outcome in the averaged model and are therefore represented with larger coefficients in the averaged model, a process not achievable with null hypothesis testing methods.

TWCC categories were demonstrated to be associated with both outcomes of death and major adverse event using null hypothesis testing methods. Direct comparison of the predictive value of TWCC compared to cell subtypes was not possible in the multi-model analysis conducted as the TWCC is a direct function of the addition of other cell types. The relative contribution of TWCC category compared to cell subtypes would be possible with a specifically designed multi-model approach although that was outside the scope of the present study. Although providing less pathophysiological insight, the development of the simplest and most accurate model for outcome in these patients would benefit from the quantified comparison of this relationship.

The null hypothesis testing sections of this thesis demonstrate complex interactions between the circulating cells, disease severity, traditional risk factors and medication use in the population with peripheral arterial occlusive disease. The nature of these interactions is demonstrated to have strong effects on the significance and strength of reported effect when tested using null hypothesis testing methods. The inconsistency of variable adjustment in published literature[223] makes direct comparison and meta-analysis of published literature problematic and standards for minimal variable adjustment that include disease severity should be considered in addition to standardised definitions and methods for assessing and recording risk factors previously discussed.[223]

Proportionality of variables is inconsistently reported in the population with peripheral arterial occlusive disease although the circulating lymphocyte count exhibited a significant cox.zph in most of the Cox proportional hazards analysis with the outcomes of death and major adverse event. Although null hypothesis methods exist to examine association in systems with proportional variables, particularly the use of time dependent variables, these were not considered necessary to establish precedent for inclusion in the multi-model analysis of this study. Such techniques may be considered in future studies where uncertainty exists about which variables to include in future multi-model analysis. The Information-Theoretical statistical approach offers an alternative for examining correlated variables that should be considered more informative in future analysis of this population.

The method of application of the averaged models for outcome in patients with peripheral arterial occlusive disease by the clinician at the bedside or in the outpatient clinic have been described using a simple computational application for smartphone. Such an application consisting of an interface that enables the clinician to select the patient risk factors, clinical severity of disease and category of circulating cell counts will enable individual clinicians to be able to apply this model to individual patients with nothing more than clinical history, full blood count results and a smartphone. It has been proposed that this application should not be released for widespread use until the model has been validated among other populations of patients with peripheral arterial occlusive disease.[419] From the multi-model inference results presented in this thesis, Lymphocyte category, Neutrophil Category, Monocyte Category, clinical disease severity, patient age, gender, history of hypertension and ischaemic heart

disease should be combined in the model (Figure 9.2) they strong predictors of major adverse events and death in patients with peripheral arterial occlusive disease.

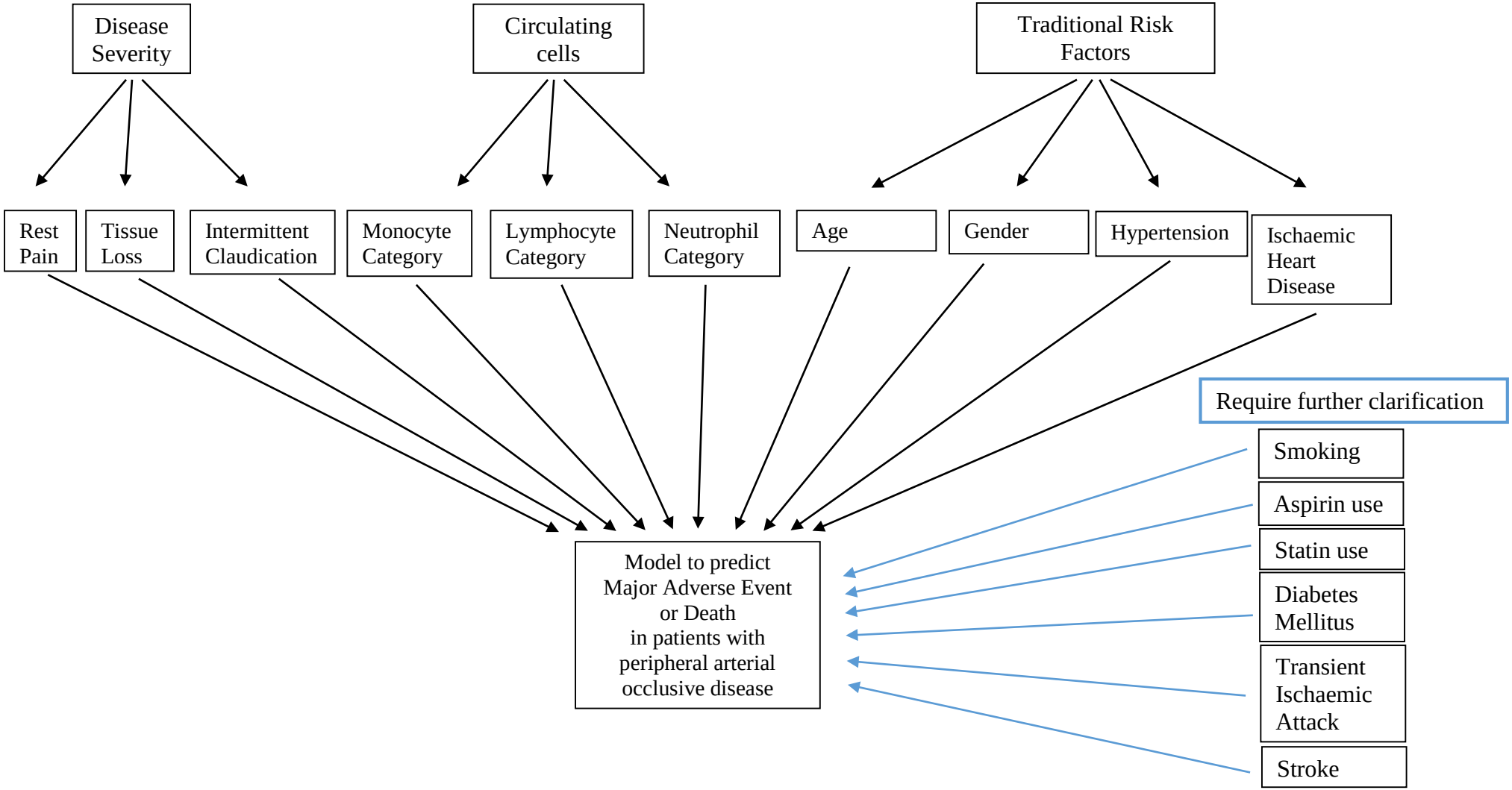

Figure 9.2: Schematic representation of multi-model outcomes showing the essential variables in each category

Multi-model analysis has revealed essential variables from each of the categories of disease severity, circulating cells and traditional risk factors for the outcomes of major adverse event and death (Figure 9.2). The relative importance of these essential variables was different in the averaged model for each outcome as discussed above but are considered essential variables to the model. Smoking status, history of Transient Ischaemic Attack, Diabetes Mellitus along with aspirin and statin use require further clarification (Figure 9.2) and would best have their role in the final model clarified by replication with another population.

From this study alone, the variables that require further clarification (shown in Figure 9.2) add meaningfully to the model and should be included. When variables are present in multiple models in the top model set with a small coefficient assigned or with the standard error including 0, it is worth considering if that variable has an interaction with another variable included in the model. For example, smoking is present in 12 of the 19 best fit models for death, with an averaged co-efficient of 0.14 and a standard error of 0.15. This may seem to be a small co-efficient for a risk factor that has been well associated with death in this population, however in the averaged model the contribution of smoking status is mostly accounted for by one or more of the other variables. Smoking status has a clear association with increased total white cell count,[22,420,421] predominantly neutrophil count,[422] therefore in the averaged model for death the contribution of smoking to the model is mostly accounted for by the circulating cell counts. Smoking is not completely replaced by these surrogate markers in the developed average model, therefore requires inclusion but it also is not able to predict the outcome of death as well as the circulating counts which is why their respective weights are larger. To the authors knowledge this is the first time these variables have been quantitatively compared in this way.

The variables of aspirin, statin use and transient ischaemic attack were only present in 1 of the 19 top models for death and their inclusion in the averaged model is to ensure that an important weak variable is not discarded. With the averaged model co-efficient for these three variables approaching 0 it is unlikely these variables offer any substantial contribution to this model for the outcome of death that is not already accounted for by another variable/s, although which variable/s remains unclear and will require further investigation. Aspirin use has been associated with lower total white cell count values in four non-randomised studies of cardiovascular risk[86,88,87,118] although neither those trials nor this study were designed to investigate that relationship. Until these relationships are clarified or these variables are excluded from the best model set by replication in a larger population they add meaningful information to the model and should remain.

The proposed handheld application of these models may be used to improve individual patient compliance with lifestyle and risk factor modification[133] including targeted smoking cessation, weight loss and dietary modifications,[135] exercise therapy to increase tolerated walking distance,[423-425] and improve individual patient compliance with drug therapy including antiplatelet, antihypertensive, statin[426] and ACE[135] that have been shown to improve outcomes in patients with peripheral arterial occlusive disease.[83,85,135,136] Optimising medical management of patients identified as high risk or with clinically advanced disease has important implications in the prevention of subsequent major adverse events and death for these patients.[427] Once prospectively validated this model may be used to risk stratify patients and allow selection of the most appropriate vascular intervention for the individual patient.[137] Risk stratification is important in this population as early successful intervention is crucially

important to reduce the risk of major adverse event, minimise long term disability and improve quality of life, but exposing patients who have high mortality risk to ineffectual operations or interventions has serious ethical and resource implications.[152] The accuracy of the instrument that is used to aid in such decision making is therefore of crucial importance.[152]

Future application of this modelling technique to include the larger population including people asymptomatic of vascular disease has the potential to identify patients at high risk of major adverse cardiovascular event in the future who would benefit from early aggressive risk factor management.[428] The low detection rates for peripheral arterial occlusive disease result in problems identifying the large burden of asymptomatic disease in the primary care setting[5]. This is problematic from a public health perspective as asymptomatic patients have similar cardiovascular risk to claudicants. Expanding this study structure and subsequent outcome model development to include asymptomatic patients would possibly require the inclusion of the Ankle Brachial Index to quantify disease severity of asymptomatic patients.[429,430] Further research would potentially offer a handheld application to identify patients at high risk in the primary care setting who would benefit from early risk factor modification and result in the proven reduction in morbidity and mortality for this population.[12]

The outcome models developed in this study should be used to guide further research direction into the pathophysiological basis of peripheral arterial occlusive disease as multi-model analysis results may be used to infer the key biological processes determining an outcome.[132] Multi-model analysis is also suggested as an ideal tool to objectively compare other potential predictors of outcome including Ankle Brachial Index and other circulating biomarkers with outcome in the population of patients with peripheral arterial occlusive disease. Following

validation the developed models may also be used to identify interventions that may offer immediate survival advantage for this patient group.

9.1. Limitations

Direct causation was not established with this study and whether the circulating cell counts are causative or reactive to the underlying inflammatory disease of atherosclerosis requires further investigation. While the underlying processes and causal relationships are being investigated there is clinical potential to exploit the association of circulating cells with major adverse event and death. Patients with peripheral arterial occlusive disease identified as being at high risk of sustaining major adverse event or death should be offered targeted interventions while the pathophysiological causal relationship is elucidated.

This study design was observational in nature and therefore inherently subject to selection bias. The population recruited did not represent all peripheral arterial occlusive disease patients in the community as it was a hospital acquired sample. Caution should therefore be used in extrapolating these results to different populations, particularly including asymptomatic patients. The study sample was representative of patients with symptomatic peripheral arterial occlusive disease who were referred for intervention, as represented by the skewed nature of the freedom from peripheral revascularisation data (Figure 6.7, Table 6.6). The study did not intend to assess or infer applicability to patients with asymptomatic peripheral arterial occlusive disease although these patients have been described to have a similar cardiovascular risk to claudicants[21,74] and expansion of the investigated population to include patients with asymptomatic peripheral arterial occlusive disease is suggested as a further direction of study.

The sample size of this study did not allow adequate power to investigate the cardiovascular endpoints of heat attack, stroke or major amputation individually and would require replication in a larger study population to investigate these outcomes.

This study used patient report of smoking status which although has previously been shown to be a valid measure,[342] a more recent systematic review has shown self-reporting underestimates the true prevalence of smoking although the degree is dependent on the population studied.[431] In order to better define this relationship the author would recommend an objective measure of smoking habit (serum or saliva cotinine) be applied in future research to objectively define this variable.

The efficacy of treatments, both medical and surgical and influence on cardiovascular events was outside the scope of this study, although the implications of both medical and surgical management have been raised as important directions for further research. The alteration of medical treatment after the sampling of "baseline study bloods" was recorded but not examined within this study design.

9.2. Suggested objectives of future research

Future investigations should replicate this study design with multi-model analysis of larger data sets in similar populations of patients with peripheral arterial occlusive disease to refine the predictive ability of the developed models. Multi-model comparison of TWCC with cell subtypes for the outcome of major adverse event and death should be considered. A broader population of peripheral arterial occlusive disease patients including asymptomatic patients may aid in identifying the patients at highest risk of major adverse event who would benefit most from the lifestyle changes and medical management discussed above. The individual endpoints of heart attack, stroke, major amputation and requirement for peripheral revascularisation would require larger study populations than in this study to power investigations of those endpoints.

The Information-Theoretical approach and in particular multi-model analysis should also be applied to other manifestations of atherosclerosis in particular coronary artery disease and aneurysmal disease to enable the creation of robust predictive outcome models for these distinct populations. This may aid in directly comparing the value of existing predictors of outcome, identifying further predictive markers, increase understanding of underlying pathophysiology to direct further research and identify potential targets for therapy whilst better investigating differences in these populations.

Treatment efficacy, both medical and surgical, and their influence on the outcomes of major adverse event and death warrant further investigation. Investigation to define the protective effect of hypertension identified in this study, or defer this benefit to the treatment applied for hypertension within this population may give further insight and add strength to the known benefits of management of this risk factor in peripheral arterial occlusive disease patients. The effect of revascularisation on both circulating cell counts and morbidity and mortality would require a larger study population to provide the power to investigate these endpoints. White blood cell function and behaviour including deformability and adhesion properties and how this varies between the groups is another direction of future research to investigate potentially modifiable risk. The Information-Theoretical approach would be ideal to account for the complex variable interactions within these analysis. Comparison of different therapies, for example exercise therapy vs revascularisation could also be done using these statistical techniques. Developed models for outcome of major adverse event and death will be better able to identify underlying biological processes and should be used to guide future pathophysiological and therapeutic research into peripheral arterial occlusive disease.

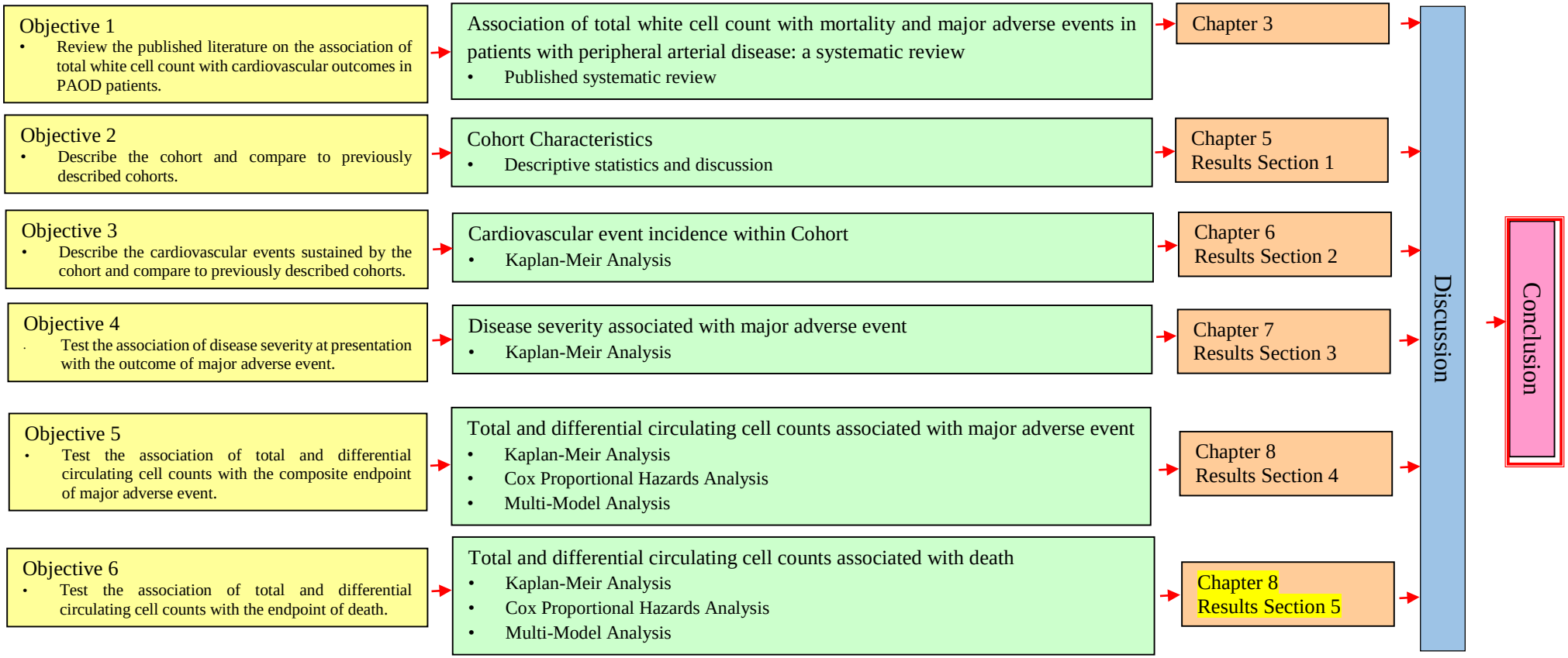

Figure 9.2: Schematic overview of thesis with red box highlighting current position within document- Conclusion

10. Conclusion

Multi-model averaging is a relatively new statistical paradigm based on the Information-Theoretic approach[132] and is the preferred statistical method for analysing data from complex biological systems needing to account for multiple co-variables of modest effect that interact to produce clinically relevant outcomes including major adverse events and death for patients with peripheral arterial occlusive disease. This is the first study to employ multi-model averaging to the population of patients with peripheral arterial occlusive disease. This study has demonstrated that circulating cell counts of monocytes, lymphocytes and neutrophils together with disease severity at presentation, hypertension and age are more important in models to predict the outcome of major adverse event and death than other traditional risk factors.

Rest Pain was the strongest predictor of major adverse event and Tissue Loss was the strongest predictor of death in the respective best averaged models. Disease severity should continue to be reported and accounted for when examining outcomes in patients with peripheral arterial occlusive disease.

High Monocyte Category exerted the strongest effect of the circulating cell types in the averaged model for major adverse event almost 1.5 times the effect of ischaemic heart disease although corroborating evidence for the association of monocytes with the composite outcome of major adverse event in peripheral arterial occlusive disease patients is lacking. High Monocyte Category was not as strong a predictor for the outcome of death, possibly indicating that is a stronger predictor of heart attack or stroke in this population than death alone.

High Neutrophil Category in the best averaged model for death had an effect size twice that of the Mid Neutrophil Category and the High Lymphocyte Category had the largest negative effect for the outcome of death, 3.25 times that of the Mid Lymphocyte Category, both of which had a larger effect than the traditional risk factors of smoking, diabetes, transient ischaemic attack, stroke and Aspirin and Statin use. Both the High Neutrophil Category and High Lymphocyte Category (negative/protective) exhibited a larger effect for the outcome of death than that of the best traditional risk factor predictor hypertension. Lymphocyte Category was the circulating cell in the largest number of models in the top model set for the outcome of major adverse event and was in all of the top model set for the outcome of death. The protective association of Lymphocytes for major adverse event and death in peripheral arterial occlusive disease patients deserves further research. The inclusion of both Lymphocyte Category and Neutrophil Category together provides better modelling of the outcome major adverse event and death than the combined Neutrophil-Lymphocyte ratio and this finding should be considered in future study design.

Hypertension was the strongest and most consistent traditional risk factor in both models for death and major adverse event with a negative or protective predictive value for both endpoints. This effect was possibly due to the protective effects of hypertensive treatment with a large proportion of the study population on antihypertensive treatment. This study was not designed to investigate the relative protective effect of antihypertensive medications although this is a suggested direction of further research.

Multi-model inferencing should be applied to larger data sets of patients with peripheral arterial occlusive disease to provide valuable information about the association of cell counts with

outcomes in this population. Once the outcome models for major adverse event and death are refined and prospectively validated, not only will they predict the likelihood of major adverse event and death for individual patients with peripheral arterial occlusive disease to tailor treatment selection, but drive future pathophysiological research and generate potential treatment options.

11. References

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Appendix A: James Cook University Ethical Approval

Appendix B: Mater Health Services North Queensland Ethical Approval

This administrative form has been removed

Appendix C: Townsville Health Service District Ethical Approval

This administrative form has been removed

Appendix D: Participant Informed Consent Form Townsville Hospital

This administrative form has been removed

Appendix E: Participant Consent Form – Mater Hospital

This administrative form has been removed

Appendix F: Participant Information Sheet – Townsville Hospital

Queensland Government
Queensland Health

Townsville Health Service District

Institutional Ethics Committee

Version 3 Approved 5/9/11
PROTOCOL NAME: The role of differences in circulating factors in the pathogenesis of vascular disease.
INVESTIGATORS:

You are being invited to take part in a research study. Please let us know if you are already taking part in any other medical studies. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with relatives, friends and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information.
The study is to further our understanding of the process of blood vessel disease, i.e. why blood vessels weaken or block. We are looking at the relationship between common differences in genes which control the level of circulating factors in the human body and the weakening and blockage process. The study will involve examining the presence of common alterations in these genes and relating it to disease in your artery. At present these genetic differences are not known to have any long term effects on yourself or your offspring. The work will not provide you with any useful information about your personal genetic background, but rather will further our understanding of the role of specific proteins in blood vessel disease and will be published in scientific journal. The blood vessel disease level will be assessed by analysis of scans you undergo as part of your clinical care including ultrasounds and CT scans.
It is up to you to decide whether or not to take part. If you do decide you will be asked to sign a consent form and given this information sheet to keep. Participation in the study is entirely voluntary and you have the right to withdraw from the study at any time. If you decide not to participate in this study you may do this freely without prejudice to any future treatment.
If you consent to participation in the study we will collect a blood sample from you and in addition if your treatment involves an operation to remove part of your diseased blood vessel we also request your permission to collect and analyse this rather than it being discarded. This will allow us to relate our studies of the circulating proteins (from your blood test) to the disease within the blood vessel (from the excised specimen). The information and samples we collect will be de-identified to maintain your privacy and stored in secure facilities. Following the operation you are normally followed up in the outpatients clinic at around six weeks and then as necessary after this. The study will not require you to make any extra visits to the clinic.
Any further information you require can be obtained from , telephone . The Ethics Committee has approved this study at the Townsville Hospital. Should you wish to discuss the study with someone not directly involved, in particular in relation to matters concerning policies, information about the conduct of the study, or your rights as a participant, you may contact the Chairperson, Townsville Health Service District Human Research Ethics Committee, PO Box 670, Townsville QLD 4810; Phone: (07) 47961140.
INVESTIGATOR CONTACT NAME:
INVESTIGATOR CONTACT TELEPHONE NO.
DATED:
SIGNATURE OF CONTACT INVESTIGATOR:

Appendix G: Participant Information Sheet Mater Hospital

MATER MISERICORDIAE HOSPITAL TOWNSVILLE LIMITED

PROTOCOL NAME: The role of differences in circulating factors in the pathogenesis of vascular disease.
INVESTIGATORS:

You are being invited to take part in a research study. Please let us know if you are already taking part in any other medical studies. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with relatives, friends and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. The study is to further our understanding of the process of blood vessel disease, i.e. why blood vessels weaken or block. We are looking at the relationship between common differences in genes which control the level of circulating factors in the human body and the weakening and blockage process. The study will involve examining the presence of common alterations in these genes and relating it to disease in your artery. At present these genetic differences are not known to have any long term effects on yourself or your offspring. The work will not provide you with any useful information about your personal genetic background, but rather will further our understanding of the role of specific proteins in blood vessel disease and will be published in scientific journal.

It is up to you to decide whether or not to take part. If you do decide you will be asked to sign a consent form and given this information sheet to keep. Participation in the study is entirely voluntary and you have the right to withdraw from the study at any time. If you decide not to participate in this study you may do this freely without prejudice to any future treatment.

If you consent to participation in the study we will collect a blood sample from you and in addition if your treatment involves an operation to remove part of your diseased blood vessel we also request your permission to collect and analyse this rather than it being discarded. This will allow us to relate our studies of the circulating proteins (from your blood test) to the disease within the blood vessel (from the excised specimen). The information and samples we collect will be de-identified to maintain your privacy and stored in secure facilities. Following the operation you are normally followed up in the outpatients clinic at around six weeks and then as necessary after this. The study will not require you to make any extra visits to the clinic. Any further information you require can be obtained from telephone .

The Ethics Committee has approved this study at the Mater Hospital. Should you wish to discuss the study with someone not directly involved, in particular in relation to matters concerning policies, information about the conduct of the study, or your rights as a participant, you may contact the Executive Officer of the Ethics Committee at the Mater Hospital (47274444).

INVESTIGATOR CONTACT NAME:
INVESTIGATOR CONTACT TELEPHONE NO. DATED: 16/7/05
SIGNATURE OF CONTACT INVESTIGATOR:

Appendix H: Copyright Declaration

Email correspondence confirming the right to include the publication below in this thesis.

Martin D, Wallace D, Crowe M, Rush C, Tosenovsky P, Golledge J. Association of total white cell count with mortality and major adverse events in patients with peripheral arterial disease: a systematic review. *European Journal of Vascular and Endovascular Surgery.* 2014;47(4):422-432.

Appendix I: Classification of peripheral arterial occlusive disease

Fontaine		**Rutherford**			**This Study**
Stage	Clinical	Grade	Category	Clinical	
I	Asymptomatic	**0**	0	Asymptomatic	Excluded from this study
IIa	Mild Claudication >200m	**I**	1	Mild Claudication	Intermittent Claudication
IIb	Moderate to Severe Claudication <200m	**I**	2	Moderate Claudication	
		I	3	Severe Claudication	
III	Ischaemic Rest pain	**II**	4	Ischaemic Rest pain	Rest Pain
IV	Ulceration or Gangrene	**III**	5	Minor tissue loss	Tissue Loss
		III	6	Major tissue loss	

The Fontaine classification, initially published in 1954[345] classifies the severity of vascular insufficiency into 4 stages is presented beside the Rutherford classification[344] which further categorised the moderate and severe claudication as well as minor tissue loss from major tissue loss. The classification scheme of this study is demonstrated as directly arising from the Fontaine classification system with depiction of how the Rutherford categories were applied to this study population.