Queen Margaret University

EDINBURGH

DEVELOPMENT AND VALIDATION OF A PROGNOSTIC MODEL FOR STUMP HEALING IN MAJOR LOWER LIMB AMPUTATION

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
2016

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List of Abbreviations

ABI	Ankle-Brachial Index
ABPI	Ankle Brachial Pressure Index
ACE	Angiotensin Converting Enzyme
ADA	American Diabetic Association
ADVANCE	Action in Diabetes and Vascular Disease: Preterax and Diamicron
AKA	Above Knee Amputation
BKA	Below Knee Amputation
BMI	Basal metabolic index
BP	Blood pressure
CHD	Chronic Heart Disease
CI	Confidence Interval
CKD	Chronic kidney disease
CLI	Chronic Limb Ischaemia
CRP	C- Reactive Protein
CTGF	Connective Tissue Growth Factor
DAG/PKC	Diacylglycerol/Protein Kinase C
DCCT	Diabetes Control and Complications Trial
DCCT	Diabetes Control and Complications Trial
DFU	Diabetic Foot Ulcer
DHEA	Dehydroepiandrosterone
DKA	Diabetic Ketoacidosis
DM	Diabetes mellitus
DPP	Di-Peptidyl Peptidase
DR	Diabetic Retinopathy

DVT	Deep vein thrombosis
EDIC	Epidemiology of Diabetes Interventions and Complications
EGFR	Estimated glomerular filtration rate
EPC	Endothelial progenitor cells
ESRD	End stage renal disease
ESRD	End Stage Renal Disease
GAD	Glutamic Acid Decarboxylase
GDM	Gestational Diabetes Mellitus
GLP	Glucagon-Like Peptide
HbA1C	Glycosylated Haemoglobin
HDL	High density lipoprotein
HHS	Hyperosmolar Hyperglycaemic State
HIF-1α	Hypoxia Inducible Factor 1α
HLA	Human Leukocyte Antigens
HNF	Hepatocyte Nuclear Factor
HTN	Hypertension
IDSA	Infectious Disease Society of America
IFG	Impaired Fasting Glycaemia
IGT	Impaired Glucose Tolerance
IL-1-β	Interlukin-1- β
IL-6	Interlukin-6
INR	International normalization ratio
IWGDF	International Working Group on the Diabetic Foot
K +	Serum potassium
LDL	Low-Density Lipoprotein

LEA	Lower Extremity Amputation
MDFT	Multidisciplinary Footcare Team
MODY	Maturity-onset Diabetes of the Young
MRSA	Meticillin-Resistant Staphylococus.aureus
Na+	Serum sodium
NASDAB	National Amputee Statistical Database
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NPWT	Negative Pressure Wound Therapy
OHA	Oral Hypoglycaemic Agents
OR	Odds Ratio
PAD	Peripheral Arterial Disease
PDR	Proliferative Diabetic Retinopathy
PMN Leukocyte	Polymorphonuclear leukocyte
PPV	Pars Plana Vitrectomy
PT	Prothrombin time
PVD	Peripheral vascular disease
QOF Data:	Quality and Outcomes Framework
RAAS	Renin-Angiotensin Aldosterone System
ROS	Reactive Oxygen Species
ROC	Receiver Operating Curve
SDF 1-alpha	Stromal cell Derived Factor 1-alpha
SIGN	Scottish Intercollegiate Guidelines Network
TC	Total cholesterol
TG	Triglyceride

TNF-α	Tissue Necrotic Factor- α
UKPDS	United Kingdom Prospective Diabetes Study
UKPDS	United Kingdom Prospective Diabetes Study
VAC	Vacuum Assisted Closure
WCC	White Cell Count
WESDR	Wisconsin Epidemiologic Study of Diabetic Retinopathy
WMD	Weighted Mean Difference

Acknowledgements

To

Dr Derek Santos (Senior Lecturer & Programme Leader, QMU) for guiding me through the difficult times with his invaluable advice and suggestions.

Dr Tom Carline (Retd Senior Lecturer & Programme Leader, MSc Diabetes, QMU) for helping me through the intial part of the project.

Mr Zahid Raza (Consultant Vascular Surgeon & Honorary Senior Lecturer) for his guidance and support throughout the duration of the project.

Mr Robert Rush (Medical statistician, QMU) for helping me see sense in the figures and the statistics of the project.

Ms Evelyn Weir (Lecturer, QMU) for her time to proof read the thesis.

Prof.Davidson for her support through the latter half of the project.

Staff in the vascular ward in the Royal Infirmary of Edinburgh and Astley Ainslie Hospital for their support.

Staff in Queen Margaret University for their encouragement.

My parents who remain my pillars of strength.

My sisters who are my backup system.

Lorna Germain and Ian King (Laboratory, RIE) thanks for sharing with me your passion and knowledge in medical lab Royal Infirmary of Edinburgh.

My wife for her unwavering support, sacrifice and dedication in my cause and putting up with me through the duration of the project.

And finally, my little angels for their smile that is enough to make me forget the stress that comes with a PhD project.

ABSTRACT

Introduction

Stump healing is essential in patients with a lower limb amputation in order for them to mobilize again. Little research has been being done on factors affecting stump healing. The aim of this paper is to explore the effect of haematological makers as well as patient characteristics on stump healing after patients have undergone an amputation procedure. In addition, a practical model regarding factors that affect stump healing was developed.

Methods

Patients who underwent a major lower limb amputation (above knee and below knee) at the Royal Infirmary of Edinburgh from the period of 2006 to 2009 were included in this study. A prognostic model utilizing backward stepwise logistical regression was developed to measure the probability of lower limb stump healing. The relationship between the dependent and independent variables was identified using univariate and multivariate logistic regression. Hosmer and Lemeshow goodness of fit test and Receiver Operating Curve (ROC) was used in order to measure the effectiveness of the model. The model was validated with the prospective data of 100 patients that had undergone major lower limb amputation from the year 2010 and 2011 in Royal Infirmary of Edinburgh prospectively.

Results

In this study healing of the stump as defined was achieved in sixty three percent (63%) of patients. Univariate analysis found seven variables to be associated with lower limb stump healing (type of amputation, gender, hypertension, smoking, serum sodium, serum creatinine and serum High Density Lipid cholesterol (HDL)). A further four variables (age, diabetes

mellitus, white cell count and Prothrombin Time) were added to the model secondary to their strong clinical association with the stump healing. Three variables, namely serum sodium, serum creatinine and serum High Density Lipid cholesterol were identified which influenced stump healing. Patients with normal serum sodium were 75% more likely to have lower limb stump healing compared to that of patients with abnormal serum sodium (odds ratio [OR] 1.756; 95% confidence interval [CI] 1.048-2.942). Patients with normal serum creatinine were 66% more likely to have their stump healed (OR 1.664; 95% CI 0.94 to 2.946). The healing rate of patients with a normal level of serum High Density Lipid cholesterol was 75%, in contrast to patients with an aberrant level of serum High Density Lipids cholesterol (OR 1.753; 95% CI 1.061 to 2.895). The effectiveness of the retrospective stump-healing model was demonstrated by the area under the Receiver Operator Curve (0.612), which was supported by the Hosmer and Lemeshow goodness-of-fit test (p=0.879). In the prospective study, the model's discriminatory power was verified by the area under the Receiver Operator Curve (0.584) and Hosmer and Lemeshow goodness-of-fit test (p>0.05).

Conclusion

Serum sodium, serum High Density Lipid cholesterol and serum creatinine have a strong correlation with lower limb stump healing. However, serum sodium and serum High Density Lipid cholesterol secondary to multiple co-morbidities in this cohort group could be altered secondary to disease pathology itself. Further clinical research is necessary to evaluate the association of the risk factors with lower limb stump healing.

CHAPTER 1

INTRODUCTION

1.1 Lower limb amputation

Lower limb amputation is one of the oldest surgical procedures and is performed frequently. Though lower limb revascularisation techniques have vastly improved, an amputation is still commonly performed secondary to advanced peripheral arterial disease (Allie *et al.* 2005).

In contrast to patients without diabetes mellitus, patients with diabetes mellitus are twice as likely to develop several complications which affect the lower limbs, such as peripheral vascular disease, peripheral neuropathy, ulceration and amputation (Gregg *et al.* 2004). According to Mountford *et al.* (2007) the risk of lower limb amputation alone is almost 30 times higher in patients with diabetes mellitus than in the normal population.

According to Kazmers *et al.* (2000), the mortality rate in the first month following amputation showed a significant variation, from 8% to 23%. High peri and post-operative mortality and morbidity rates among patients suffering from peripheral vascular disease, who underwent a lower limb amputation were also noted by Aulivola *et al.* (2004). Subramaniam *et al.* (2005) argued that the higher the location of the amputation, the higher is the mortality rate in the period immediately after the amputation surgery, potentially indicating the severity of advanced vascular disease.

Hospital admissions for foot ulceration have increased over the past decade as about 15% of the people with diabetes mellitus go on to develop foot ulceration (Frykberg *et al.* 2006). In the United Kingdom, the 56% prevalence of leg amputation as a result of vascular complication in 1998/99 increased to 74% in 2011-12 (United National Institute for Prosthetics & Orthotics Development annual report. 2012). The number of people who have undergone lower limb amputation in the United Kingdom is approximately 5000 in 2011-12 and out of which 50% of referrals to prosthetic devices have had a below knee amputations as reported by the Limbless

Society formally known as the Amputee Statistical Database for United Kingdom (United National Institute for Prosthetics & Orthotics Development Annual Report. 2012). According to National Diabetes Audit (2011/12), dysvascularity was the most common indication accounting for 75% of all lower limb amputation, half of which were secondary to diabetes mellitus. Though the report largely reflected the entire population in the United Kingdom, major geographical differences in lower limb amputation rates have been noted, possibly depicting differences in decision making due to local clinical guidelines and policies and their delivery, although ethnic factors may also contribute (National Amputee Statistical Database for the United Kingdom. 2007).

1.2 Stump healing in lower limb amputation

The process of stump healing of an amputee is important because it enables the patient to regain limb function with the use of a prosthesis. In addition to the type of treatment administered, the stump healing process is also influenced by the characteristics of the wound and the condition of the patient. There is a major variation in the healing rates of amputations, depending on the level where they were performed (National Amputee Statistical Database for the United Kingdom. 2006). Above-knee amputations have been attributed a 70%-90% healing rate, whilst below-knee amputations have a healing rate of 30%-92%, with a 30% likelihood for additional amputation procedures (Dormandy *et al.* 1999). The variation in healing rates may be due to lack of evidence based guidelines and lack of patient selection for the right. Many clinicians agree that the ultimate goal of caring for a patient with a diseased lower extremity is to maximize their quality of life by preserving their independence via their ability to ambulate (Pell *et al.* 1993). Multiple studies have documented the increased rehabilitation rate in below-knee amputation (BKA) vs above-knee amputation (AKA) patients, with more than 65% of below knee amputation patients ambulating with prostheses (Aulivola *et al.* 2004). In contrast,

less than one third of patients with above-knee amputation are likely to rehabilitate with the use of a prosthesis. However, it could be argued that the outcomes regarding quality of life improvement could be related to other factors including pain sensation, prosthetic factors, and psychosocial well-being (adaptation to amputation, prosthesis and body image). But in a survey of 44 patients with lower limb amputation investigating prosthesis satisfaction, body image, and phantom pain, Murray *et al.* (2002) found that higher levels of prosthesis satisfaction were significantly correlated with lower levels of body image disturbance. But with a below-knee amputation procedure however, there is a greater risk of non-healing of the stump resulting in a further procedure either at the same or higher level. Patient selection, given this variability in outcomes, therefore becomes important. This thesis provides new evidence to facilitate with patient selection and aid clinicians in decision making with regards to when it is best to proceed with a below-knee amputation and achieve a decreased risk complication and where a successful outcome is more likely.

Izumi *et al.* (2006) emphasized that the success of an amputation depends on wound treatment as well as patient awareness of the implications of such a surgical procedure. According to the studies conducted by Canavan *et al.* (2008) the prevalence of leg amputations can be reduced by up to 78% by employing an adequate multidisciplinary diabetes mellitus treatment. To obtain the best results after an amputation, a meticulous approach to the preoperative assessment and surgical technique is necessary.

1.3 Research problem and aims of the study

1.3.1 Rationale of the study

Lower Extremity Amputations (LEA) is a major source of morbidity and mortality in patients with diabetes mellitus (Hambleton *et al.* 2009). According to estimates, every half a minute a

patient with diabetes mellitus loses a leg due to an amputation (Boulton *et al.* 2005). The life expectancy rate among individuals who have been subjected to major amputation procedures is low and can be compared to survival rates in cancer patients (5-year mortality rates up to 55% vs 73% in colon cancer vs lower limb amputation) (Armstrong *et al.* 2005). According to Schofield *et al.* (2006), people with diabetes mellitus also had a 55% greater risk of death than those without the disease. Diabetes foot disease requires patients to spend a considerable period of time in hospital, which contributes to 25% of the hospital expenditure for diabetes mellitus (Canavan *et al.* 2008). There is a growing necessity for interdisciplinary treatment for amputations secondary to diabetes mellitus, as the number of people with diabetes mellitus continues to increase.

The proportion of the elderly in the population of the United Kingdom is considerable, signifying that an increasing number of patients with diabetes mellitus of advanced age will go on to require some form of lower limb amputation surgery (National Amputee Statistical Database for the United Kingdom. 2006). This poses questions about the viability of subjecting older patients to surgical procedures, given the reduced healing and the co-existence of multiple conditions such as diabetes mellitus and peripheral vascular disease. Most patients in the United Kingdom referred for an amputation procedure are 75 years of age or older (United National Institute for Prosthetics & Orthotics Development annual report. 2011). The increased age of this group increases the risk of developing amputation-related complications and demands the creation of more efficient treatment to ensure the survival of these patients.

Healthcare professionals are faced with the challenge of assessing the different risk factors and deciding which one of them have a greater influence on the stump healing rate. There are currently an insufficient number of studies regarding factors effecting lower limb amputation. The available studies exhibit wide variation in structure and outcome. In addition, the related

literature shows discrepancies regarding methods of patient selection, surgical procedures and post-operative implications. The variation exhibited by such studies makes it difficult to reach a conclusion about the way stump healing affects limb rehabilitation. It is imperative that further studies are conducted to enable the creation of a set of guidelines regarding factors contributing to the healing of the stumps following leg amputation (Nawijn *et al.* 2005).

The biomarkers used in this study were chosen because they were readily available given the scope of the study. A good biomarker is one that is relevant to the study, is cost effective, easily reproducible, has a high sensitivity, specificity and validity proven towards that disease and least prone to measurement errors and bias. The markers used in this study are factors that play a role in healing of a diabetic foot ulcer as noted in several studies and play a role in the pathophysiological pathways of peripheral vascular disease and diabetes which are the main causes for a lower limb amputation surgery. These biomarkers are easy to use, readily available for a surgeon/physician on a day to day basis for decision making, are cost effective and do not require any equipment or any expertise to use or interpret. The blood markers used are readily available via the laboratory in any hospital setting almost daily for any inpatient who has regular blood check for his illness.

1.3.2 Research question

What are the important blood markers and patient factors that can be helpful in determining lower limb stump healing before surgery?

1.3.3 Aims of the study

The aim of the present study was to conduct an evaluation of the potential predictive factors of the healing process of lower limb amputation in people with diabetes mellitus suffering from advanced atherosclerosis. The identification of such factors will not only enhance the quality

of life of the patients but will also be economically and clinically beneficial, as it could enable an efficient management of the healthcare budget.

1.3.4 Objectives

- To retrospectively explore the potential influence of blood markers and patient factors (risk factors, kidney function profile, coagulation profile, lipid profile and infection markers) on stump healing of patients with diabetes mellitus who have undergone lower extremity amputation surgery at the Royal Infirmary of Edinburgh.
- To prospectively explore the influence of blood markers and factors, as classified by kidney function tests, coagulation profile and lipid profile, risk factors, and infection markers on healing of the stump of patients who have undergone lower limb amputation surgery at the Royal Infirmary of Edinburgh and to develop and validate a prognostic model for the prediction of lower limb stump healing.

1.3.5 Outline of the thesis

1.3.5.1 Chapter 2 (Literature Review)

This chapter provides up-to-date evidence on aetiology, predisposing factors, classification of diabetes mellitus and its complications. It discusses risk factors and pathogenesis of people with Diabetic foot ulcer as a pre-amputation state. It also discusses the etiopathogenesis and latest trends in lower limb amputation. Finally, relationship of patients' factors, blood markers and stump healing post major lower limb amputation is discussed.

1.3.5.2 Chapter 3 (Methods)

This chapter provides information about the retrospective and prospective part of the study. In addition, information about the research procedures including recruitment, inclusion/exclusion

criteria and details of blood markers is mentioned. Data collection and the statistical method adopted is presented.

1.3.5.3 Chapter 4 (Results)

In this chapter, results are presented for both development and validation phases of the study. Data analysis on patient's demographics, univariate and multivariate analysis are reported.

1.3.5.4 Chapter 5 (Discussion)

This chapter presents the results of the developed model and relates them to the context of the literature and current practice.

1.3.5.5 Chapter 6 (Conclusion)

The key findings of this thesis are brought together in this final chapter. The summary presents the strengths, limitations and clinical implications of the research, whilst emphasising the potential offered by the model for lower limb stump healing prior to surgery.

CHAPTER 2

LITERATURE REVIEW

2. Amputation

2.1 Introduction

Amputation is a common procedure in which a part of the body or a portion of a body part is surgically removed (Marcovitch, 2005). Despite the significant advances that have been made with regards to this surgical intervention, it is still responsible for a great number of deaths, especially among older patients. In general, widespread trauma, vascular disease and tumours are the key determinants in decision-making regarding amputation. Of these three determinants, vascular disease is currently the most widely invoked reason for amputation (Dillingham *et al.* 2002). Limbs often have to be amputated in the case of individuals suffering from severe limb ischaemia for whom vascular reconstruction has failed or is untenable and in patients with diabetes mellitus with severe foot infection. Seventy percent of all amputations performed worldwide are caused by Peripheral Vascular Disease (NASDAB, 2005). According to Eardley *et al.* (2010) because patients undergoing an amputation surgery have a high mortality due to their complex comorbidities, it is important that the patients who undergo amputation are well-informed about the procedure and its outcomes in order to obtain the best results.

2.2 Types of amputation

According to International Working Group on the Diabetic Foot, (1999) a major amputation refers to any amputation above the mid-tarsal level. "Major" limb loss is defined as amputation above the elbow, below the elbow, above the knee, below the knee, or the foot. "Minor" limb loss is defined as amputation of the hand or digits (fingers or toes) (Tseng *et al.* 2007). Lower limb amputations are much more frequent than upper limb and are most commonly the result of disease followed by trauma.

2.3 Upper limb amputation

Upper limb amputations are performed infrequently and are mostly indicated by severe traumatic injuries. The location of the injury will determine the level of amputation. Preservation of extremity length is often a goal. The amputation site will have important implications on the functional status of the patient and options for prosthetic reconstruction.

Levels for Upper Limb Amputations, according to Braddom (1996) distal to proximal are,

1. Amputation of individual digits- the thumb is the most commonly amputated digit.

2. Multiple digit amputation- when more than one digits are lost.

3. Metacarpal amputation- this type of amputation involves loss of the entire hand but the wrist is still intact.

4. Wrist disarticulation- involves the loss of the hand, but at the level of the wrist joint.

5. Forearm (transradial) amputation- this type of amputation is classified by the length of the remaining stump.

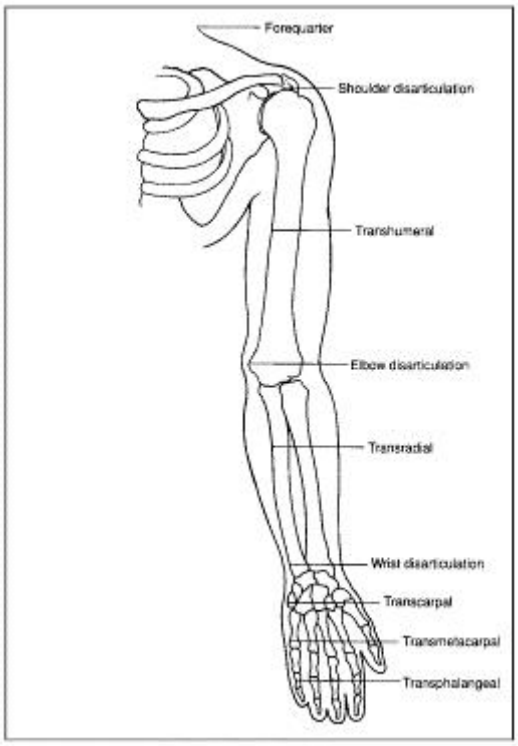
6. Elbow disarticulation- this type of amputation involves the removal of the entire forearm at the elbow.

7. Shoulder disarticulation- in this type of amputation, the shoulder blade remains. The collarbone may or may not be removed.

8. Forequarter amputation- includes removal of the shoulder blade and collarbone.

Figure 1: Levels for Amputation

Source: Braddom R.L. Physical Medicine and Rehabilitation, Philadelphia W.B. Saunders, 1996



2.4 Lower limb amputation

2.4.1 Introduction

Among amputation surgery, lower limb amputation is the most frequently performed procedure. Of all the complications of diabetes mellitus, lower limb amputation is perhaps the most debilitating, and is often associated with a high mortality rate and the likelihood of further re-amputation (Reiber, 2001).

Limb amputation has been performed since antiquity, Hippocrates being the first to have

established the stages of this surgical procedure (Murdoch *et al.* 1997). These have remained largely unchanged up to the present day, except for a number of additional developments, such as haemostasis, anaesthesia and a safer surgical environment.

The frequency of lower extremity amputations surpasses that of upper extremity amputations, being usually caused by disease and trauma. In the UK, the number of people who have undergone leg amputations has been estimated by the National Amputee Statistical Database Annual Report (2005-06) to be approximately 52,000. Ninety two percent of the referrals for prosthetic implants in the United Kingdom are individuals with leg amputations. With the prevalence of diabetes mellitus on the rise, its complications including vascular complications like lower limb amputations are also increasing. In the United Kingdom, the 56% prevalence of leg amputation as a result of vascular complications or ischaemia in 1998/99 increased to 75% in 2004/05 (NASDAB 2005). In the US, it has been estimated that the number of annual leg amputation procedures will increase to 58,000 by 2030 (Fletcher *et al.* 2002).

2.4.2 Types of lower limb amputation

According to Seymour (2002), the types of Lower Extremity Amputations organized by anatomical location, distal to proximal are:

1. Toe Amputation:

2. Transphalangeal Amputation (Toe Disarticulation)

3. Transmetatarsal Amputation

4. Lisfranc Amputation: Performed at the tarsometatarsal joint and involves disarticulation of all five metatarsals and digits.

5. Chopart Amputation: At the talonavicular and calcaneocuboid joints, it involves disarticulation through the midtarsal joint leaving only the calcaneus and talus.

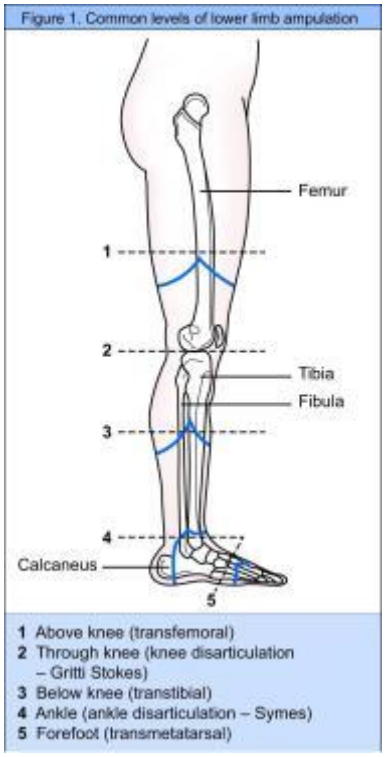
6. Syme Amputation: Ankle disarticulation in which the heel pad is kept for good weight-bearing.

7. Transtibial Amputation (BKA)

8. Transfemoral Amputation (AKA)

Figure 2: Common levels of lower limb amputation

Source: Marcovitch H, editor. Black's Medical Dictionary. London: A&C Black Publishers, 2005.



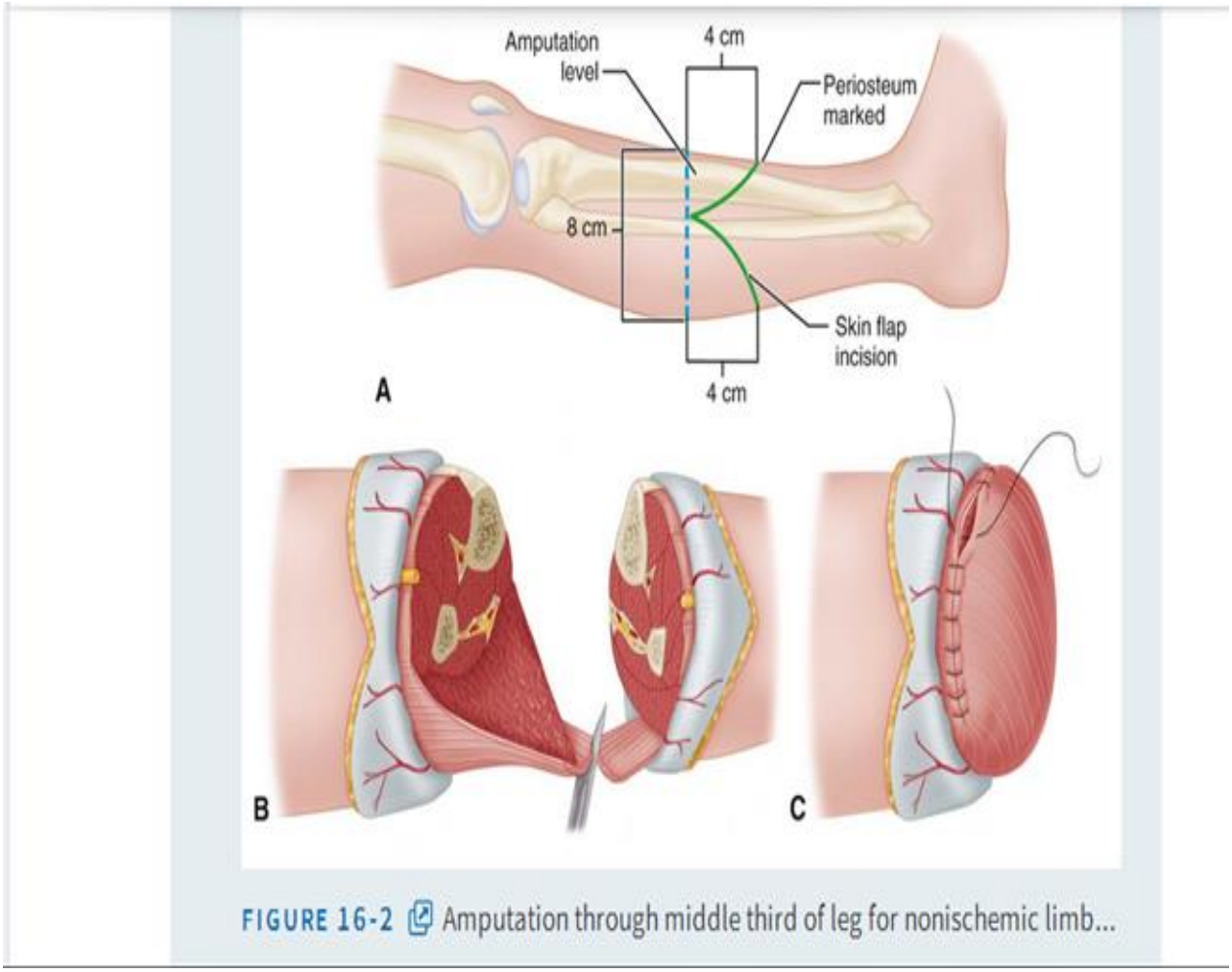
2.4.2.1 Transtibial Amputation (BKA)

Transtibial amputations make up most of all lower-limb amputations and the healing rates of below knee amputation are the benchmark to compare other amputation success including above knee amputation. This level is used primarily when the proximity of the disease process precludes a partial foot or ankle amputation.

Many techniques of transtibial amputation have been described, but the most commonly used is the long posterior flap. Burgess advocated the long posterior flap as the main advantage of transtibial amputations (Burgess, 1969). The primary goal of flap selection is to allow adequate soft tissue coverage for a tension-free closure, provide a soft tissue envelope for later prosthetic fitting, and avoid scar adhesion to the underlying bone. In this technique, the transverse anterior incision begins at the junction of the proximal two-thirds and distal one-third of the leg. Sharp corners are avoided to prevent the formation of "dog ears." All major peripheral nerves are identified and transected under tension to allow for retraction to prevent painful neuromas. The other technique used is the skew flap which is the medial flap technique. The figure below explains the process involved in selecting the level of the amputation as well as that of flap harvesting. Step A explains the general anatomy of the proposed site of the surgery. Step B explains the amputation technique. Step C illustrates the preparation of the stump.

Figure 3: Below knee amputation surgery-Level of bone section and skin flaps

Source: Canale, S. T. and Beaty, J. H. 2013. *Campbell's Operative Orthopaedics*, 12th Ed. Elsevier



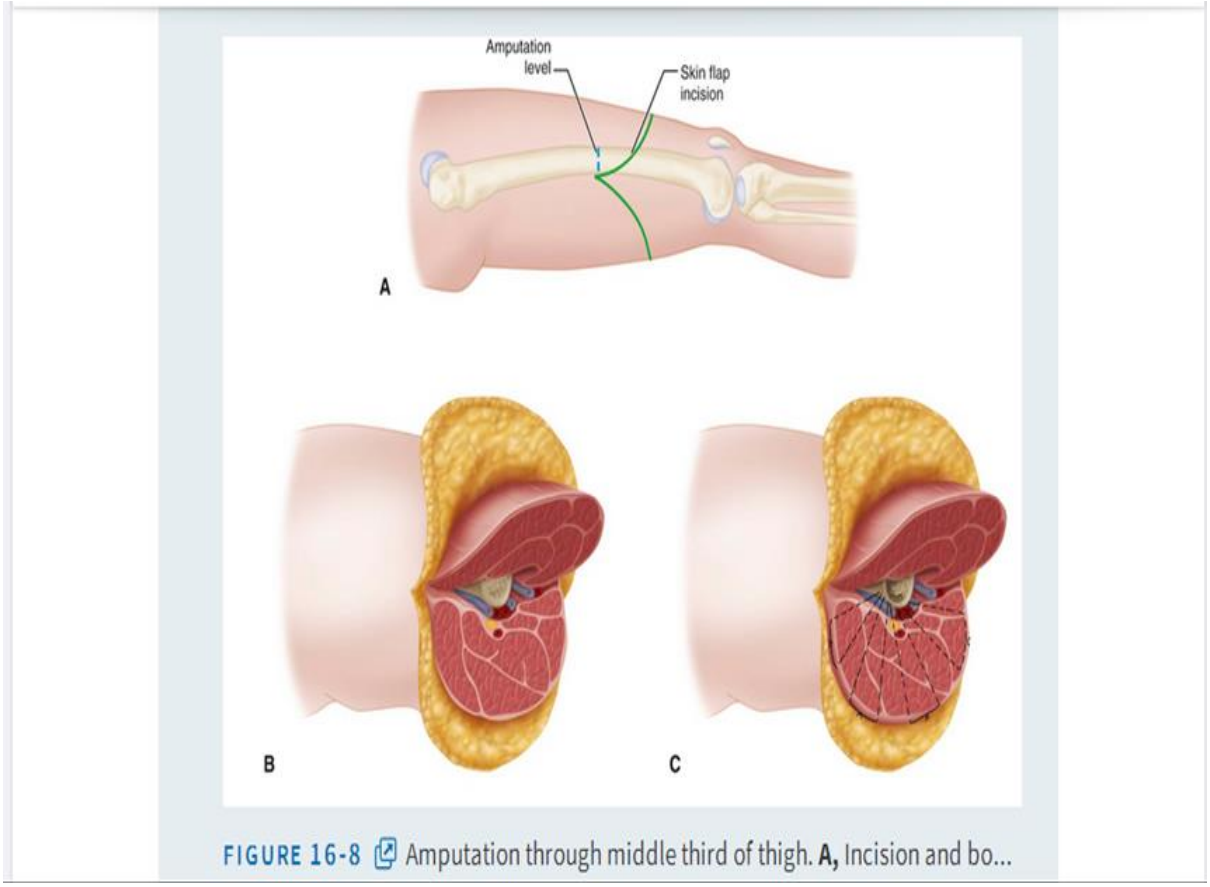
2.4.2.2 Transfemoral amputation (AKA)

More than a third of patients in the UK that are referred to prosthetics have had trans-femoral amputations (NASDAB, 2005). It is one of the common surgical procedures performed today especially with the growing prevalence of diabetes mellitus. Nowadays, this procedure is

mostly used on patients who suffer from advanced vascular disease and diabetes mellitus, who are considered to have a low healing rate for lower-level amputations. The figure below explains the process involved in selecting the level of the amputation as well as the different flap during the process of above knee amputation.

Figure 4: Above Knee Amputation surgery-Level of bone section and skin flaps

Source: Canale, S. T. and Beaty, J. H. 2013. *Campbell's Operative Orthopaedics*, 12th Ed. Elsevier



In trans-femoral amputations, a tourniquet is only used if there is a special requirement for it, and is positioned at the highest possible level on the femur and removed before setting muscle

tension. Before the operation begins, the skin flaps are carefully traced, the ones at the front being longer than the flaps at the back of the leg to ensure that the suture line will be on the dorsal side. In addition, a long flap situated on the lateral inside part of the leg can also be used, as can any form of flap which will increase possible length preservation.

There are 2 approaches to managing the muscle in the limb during amputation: myodesis and myoplasty. With a myodesis, the muscles and fasciae are sutured directly to the distal residual bone through drill holes. However, myodesis is contraindicated in patients with severe peripheral vascular disease, because the blood supply to the muscle may be compromised. Myoplasty requires the surgeon to suture the opposing muscles in the residual limb to each other and to the periosteum or to the distal end of the cut bone. Myoplasty has been suggested as a method of anchoring the muscles; however, it does not re-establish normal tension in the muscles and it does not enable proper muscle control of the thigh, diminishing muscle strength in the affected limb. Myoplasty entails the conjoining of the agonist and antagonist muscle groups over the epiphysis of the bone. This generates muscle instability and pain as a result of the movement of the femur in the muscle casing. Furthermore, the muscle casing around the end of the amputated stump can hinder the attachment of the prosthetic limb (Gottschalk, 2002).

2.5 Burden of amputation on the NHS

Despite a reduction in amputation rates worldwide, the cost implications continue to be a high for healthcare systems. This has resulted in increased diabetic foot disease often leads to serious long-term complications, putting significant socio-economic pressure on resources and health care. In the UK, the National Health Service is under enormous financial strain because of diabetic foot complications, reflected in greater outpatient costs, bed occupancy, and extended

hospitalisation. The cost of diabetic foot care alone to the UK National Health Service during 2010-2011 was £639-662 million. A proportion of 10% of the NHS budget is taken up by diabetes and diabetes-related complications account for 80% of the total cost. Li *et al.* (2010) estimated that, taking into consideration modifications in demography and the increasing rate of obesity, in the coming two decades, diabetes mellitus will come to represent 17% of the whole NHS budget, if the current cost of diabetes treatment remains unchanged (Kerr *et al.* 2012). These figures do not take account of the indirect costs to patients such as the effect on physical, psychological and social wellbeing. Social care will also require additional funding because individuals who undergo amputations require assistance in daily activities and in caring for themselves. Hence, diabetes mellitus will place an even greater strain on the health and social care system. What is more, diabetics also have significantly lower work productivity due to poor health, the cost of which has been approximated at around £9 million, though further research is needed to with corroborate this. The International Diabetes Federation has estimated that around 50% of direct healthcare costs are due to loss of work productivity in the United States (Yang *et al.* 2012).

2.6 Aetiology of lower limb amputation

The indication for performing a lower limb amputation is often multi-factorial. The International Classification of Diseases (9th Revision) has suggested a categorization of the diseases which determine leg amputation (Stroke Unit Trialists' Collaboration, 2002). According to the Amputee Coalition of America (2010), the main cause of leg amputation is vascular disease, with a rate of eight times higher than that of the second important cause of amputation which is trauma. The diseases which can lead to leg amputation are presented in Table 1. Some of the common causes include diabetes mellitus, chronic osteomyelitis and trauma.

Table 1: Aetiology of lower limb amputation

Source: International Classification of Diseases. (9th Revision) 2010-11.

Disease	***Types***
Chronic Osteomyelitis	Chronic osteomyelitis of pelvic region and thigh, lower leg, ankle, and foot.
Congenital Deformity	Transverse deficiency of lower limb, longitudinal deficiency of lower limb.
Device Infection	Vascular device, internal orthopaedic device, tissue graft, joint prosthesis
Diabetes mellitus	Diabetes mellitus type I with and without manifestations, diabetes mellitus type II with and without manifestations.
Local Significant Infection	Gangrene, actinomycotic infections, cellulitis, pyogenic arthritis, infective myositis, necrotizing fasciitis.
Lower Extremity Cancer	Malignant neoplasm of pelvic bones, sacrum, coccyx, long and short bones of lower limb, connective tissues of lower limb including hip, skin of lower limb including hip.
Previous Amputation Complication	Non-resolving infected amputation stump.
	Atherosclerosis, aortic aneurysm, venous thrombosis, arterial

Problems with Peripheral Circulation	stricture or stricture of graft, circulatory disease, venous insufficiency, organ or tissue replaced by blood vessel, gangrene, vascular complications of other vessels.
Skin Breakdown	Non-healing ulcer or decubitus ulcer of lower extremity.
Systemic Sepsis	Septicaemia, gram negative septicaemia
Trauma	Acute osteomyelitis, closed or open fractures to lower extremities, fracture of one or more phalanges of foot, trauma to AKA or BKA, open wound to lower limb, burns of lower limb, fracture of lower limb, open wound of lower limb, late effects of injuries, poisonings, toxic effects, and other external causes, crushing injury of lower limb.

According to Donohue *et al.* (2001), peripheral vascular disease is the cause of 70-80% of leg amputations performed worldwide, followed by diabetic foot ulceration infections. The table below summarizes the causes of lower limb amputation in percentages with vascular insufficiency accounting for most cases (75%). In the vascular insufficiency group, nearly half were patients with diabetes mellitus. This highlights the burden of patients with diabetes mellitus with regards to lower limb amputation (Table 2).

Table 2: Conditions that contribute to lower limb amputation in percentages

Source: National Amputee Statistical Database. National Amputee Statistical Database Annual Report, 2005-2006

Cause of lower limb amputation	***Percentage***
Vascular insufficiency	75
Neoplasia	2
Neurological disorder	2
Infection	7
Trauma	9
Other	3
No cause provided	2

The vascular insufficiency group can be broken down as follows:

Cause of lower limb amputation	***Percentage***
Diabetes mellitus	42
Non-diabetic arteriosclerosis	29
Patients for whom no additional detail was available	24
Other vascular insufficiency	5

2.6.1 Vascular insufficiency

Peripheral arterial disease (PAD) is a vascular condition typified by the presence of atherosclerotic plaques that occludes the vasculature in the lower limbs. PAD increases the risk for lower-limb amputations, but it also raises the risk of cardiovascular and cerebrovascular disease; furthermore, PAD can indicate the presence of atherosclerotic plaques elsewhere in the vasculature. According to the findings of the Framingham Heart Study, of those patients who were symptomatic for PAD, 20% co-presented with diabetes. However, since many PAD sufferers are asymptomatic, its prevalence is likely to be considerably greater than determined by the number of symptomatic patients. Indeed, more than half of those identified as having

PAD are either asymptomatic or present with atypical symptoms; approximately 30% experience pain or cramp in the legs due to claudication. The disease is present in severe form in the remaining 20% (Hiatt, 2001).

2.6.2 Diabetes mellitus

Diabetes mellitus is a well-known cause of lower limb amputation. It is discussed in further detail in the subsequent chapters.

2.6.3 Neoplasm

Amputations due to cancers of the lower limb are rare and account for under 2% of the total lower limb amputations. Tumours could be primary tumours of the lower limb or secondary/metastasis from other organs. Surgical intervention to excise growth is needed to resolve primary malignant tumours of the limbs. Excision can be localised to the tumour and sufficient margin around the site that is clear of malignancy but in some instances, may require removing the compartment in entirety. In recent years, there has been little difference between the interventions in terms of survival and disease-free states, with patients enjoying higher post-operative health and survival rates, aided by chemotherapy and/or radiotherapy (Ragnarsson *et al.* 2003).

2.6.4 Trauma

Worldwide, limb trauma is the most common reason for young, working-age people to undergo an amputation. More than 65% of trauma-related limb amputations occur in youths and adults under 45 years old. The prevalence of limb loss that is secondary to trauma is 15%. This differs significantly to the approximate 64% of amputations for adults of 65 years or more, which are attributed to vascular disease (Ebskov, 1992).

2.7 Vascular insufficiency

2.7.1 Introduction

PAD is a vascular condition typified by the presence of atherosclerotic plaques that occlude the vasculature in the lower limbs. The morbidity associated with atherosclerosis is high with the greatest burden arising from coronary artery disease and stroke, closely followed by PAD of the lower extremities. Nonetheless, the worldwide burden of PAD is considerable, as it is associated with high levels of non-fatal cardiovascular ischaemic events (heart attack, stroke and other thromboembolic events), reduced quality of life and increased mortality.

2.7.2 Epidemiology

The findings of natural history and epidemiology studies indicate that PAD affects about 30% of older individuals and increases a patient's risk of non-fatal and fatal cardiovascular ischaemia. Selvin *et al.* (2004) report that based upon the epidemiological research, the total prevalence of PAD ranges between 3–10%, rising to 15–20% in people 70 years or more.

The risk of needing lower-limb amputation is significant for diabetic patients who co-present with PAD. Although asymptomatic PAD patients are free of symptoms, they are not free of risk, with the disease increasing their vulnerability to coronary, cerebral and renal events, which in turn raise the risk of heart attacks, strokes and death. Despite being a major contributor to the mortality of patients, PAD is frequently underdiagnosed. Hirsch *et al.* (2006) conducted a conducted a multi-centre, cross-sectional study conducted at 350 primary care practices throughout the United States in 1999 with 6979 patients aged 70 years or older or aged 50 through 69 years with history of cigarette smoking or diabetes with an ankle-brachial index (ABI) of 0.90 or less as a part of PAD Awareness, Risk, and Treatment: New Resources for Survival (PARTNERS) programme, reported that about 35% of the patients were undiagnosed

despite having risk factors. (Hirsch *et al.* 2006).

2.7.3 Age and Gender predilection

Various studies have shown that PAD is a disease with a predilection for men (Aronow *et al.* 1994). However, other studies have noted that PAD was as common in women especially in those who are aged without CVD.

Albeit slight, there are gender differences in the prevalence of PAD. Whether asymptomatic or not, more men than women present with PAD; this is most noticeable in younger patients. The ratio of men to women experiencing intermittent claudication is between 1:1 and 2:1, though in severe disease states, for example, chronic limb ischaemia, the ratio has been found to rise to 3:1 (Norgren *et al.* 2007).

The Edinburgh Artery Study, which was a random sample survey of the general population, found that the occurrence of claudication in males in the 50–59 year age bracket was 2.2%, which rose to 7.7% in the 70–74 year age group (Fowkes *et al.* 1991). Most of surveys, similar trend of PAD prevalence being greater in males than females was noted, though the gap decreased with increasing age.

2.7.4 Pathophysiology of atherosclerosis

Atherosclerosis is a complex condition that has both genetic and environmental elements; it is the leading cause of disability and death in the developed world. The disease is typified as an accumulation of cholesterol, connective tissue, macrophage infiltration, over-production of smooth muscle cells and the development of thrombi (Turumen *et al.* 1999). The predisposition to developing atherosclerosis is dependent upon a number of systemic and general factors, but plaques most often develop at particular sites in the circulatory system; these include branching

points, and in small vessels, places where the artery curves (Smedby, 1996).

Atherosclerotic plaques are categorised into six categories dependent upon histological factors (Stary *et al.* 1995). Type I, which is present at birth in most people, contains mononuclear leukocytes and atherogenic lipoproteins; this typically presents with a thickening of the intima of the vascular wall. In type II, foam cells or macrophages and smooth muscle cells (SMCs) infiltrate the intima from the media. The whole plaque is described as a fatty streak, which is specific to atherosclerosis. Type III is an intermediate stage in which coarse lipid particles disturb the natural arrangement of SMCs. Type IV plaques have a large extracellular lipid core and grow outward into the arterial wall. Type V lesions are sub-categorised into types Vb and Vc. In type Vb, the lesions are generally calcified but still have a large extracellular lipid core, whereas in type Vc there is a clear increase in the accumulation of collagen and SMCs, making the lesion fibrous; type Vc lesions contain little lipid and do not show signs of calcification. Type VI plaques are those that have ruptured leading to the formation of fissures of haematomas in the lumen of the vessel. As a consequence of the rupture, the exposure of the lipid core to blood initiates platelets to aggregate, resulting in a thrombus.

2.7.5 Evolution of an atheroma

The endothelium becomes more permeable to the lipoproteins that transport lipids, such as cholesterol and triglycerides, enabling these transport proteins to bind to components of the extracellular matrix, known as proteoglycans. The affinity of heparin sulphate proteoglycan molecules for lipoproteins is strong, enabling the latter to be chemically modified. Hydroperoxides, lysophopholipids and oxysterols are products of lipid oxidation, as are aldehyde products from free fatty acids. Not only do proteoglycans make a considerable contribution to atherosclerosis, it also inhibits the proliferation of SMC (Pillarisetti, 2000).

There are no less than five different means by which lipoproteins undergo modification in the wall of the artery. The most important of these are ROS oxidation, non-oxidative glycation in diabetes mellitus and chronic uraemia. The atherogenicity of those lipoproteins that are modified by oxidation (oxLDL) exceeds that of native LDL, resulting in macrophages being recruited to the lesion. Atherosclerosis is therefore, considered an autoimmune disease as the oxLDL in the intima of the artery are determined by the immune system to be an exogenous invasion, leading to the recruitment of leukocytes to the site. Macrophages engulf the oxLDL to become foam cells. In due course, macrophages undergo apoptosis, but the engulfed lipid core persists in the intima, which then develops into the atherosclerotic plaque.

2.7.6 Role of Endothelial dysfunction

Vascular endothelium which performs multiple key functions is strategically positioned between the blood and the arterial wall. Being an endocrine organ, the endothelium regulates a number of processes, including the adhesion and migration of blood cells, coagulation, fibrinolysis, formation of NO, prostacyclins and ETs, permeation of lipoproteins and plasma proteins, proliferation of SMC, regulation of the sub-endothelial matrix and vascular tone. Furchgott and Zawadzki (1980) demonstrated that endothelium-derived NO caused vasorelaxation under the influence of acetylcholine. The endothelium also generates prostacyclin and tissue-type plasminogen, which are also effective vasodilators. Endothelium exposed to shear stress arising from blood flow turbulence and vascular stretching, such as vertebral arteries promotes its likelihood of prematurely developing atherosclerosis (Ravensbergen *et al.* 1998). The risk of atherosclerosis is promoted by ROS production in response to stimuli such as smoking, anaerobic metabolism, radiation damage and stressful conditions. In diabetes mellitus and chronic uraemia, the oxidative stress caused by ROS results in an accumulation of ‘advanced glycation end products’ (AGEPs). These AGEP peptides

initiate the activation of inflammatory cytokines and the modification of apolipoprotein B leading to a vicious cycle of atherogenesis.

The secretion of factors such as ET-1 results in increased vasoconstriction. In response to ET-1, surface adhesion molecules, such as integrins, selectins and immunoglobulins are expressed, which are ligands for the leukocytes and platelets that are chemotactically recruited to the area. Cellular adhesion molecules abundantly expressed on the surface of macrophages and endothelial are markers for atherosclerosis. Examples of these molecules that enable monocytes to adhere to the surface of the endothelium include intercellular adhesion molecule-1 (ICAM-1), P selectin and vascular cell adhesion molecule-1 (VCAM-1). Monocytes then migrate from the surface into the intima. ET-1 also triggers the mitosis of SMC and initiates vasoconstriction, which leads to the production of inflammatory cytokines and free radicals, which are released into the circulation. Furthermore, leukocyte adhesion and activation are enhanced by proinflammatory cytokines IL-1 and TNF-α at sites where the endothelium becomes inflamed or damaged. Neutrophils, granulocyte macrophage colony stimulating factor, IL-8 and plasminogen-activating factor are also generated and these in turn lead to the activation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase/stress activated protein kinase and p38 mitogen-activated protein kinase (MAPK). All three of these MAPK subtypes transduce growth factor and stress agent messages. Endothelial damage arising from ROS is promoted by activated neutrophils; their activation is greatly enhanced by the granulocyte macrophage colony stimulating factor and plasminogen-activating factor which are generated by cytokine-activated endothelial cells. The inflammatory process is exacerbated by the damage to the endothelial cells' reduced expression of plasminogen-activating factors and marked antithrombotic activities (Shoenfeld *et al.* 2000).

2.7.6.1 Role of inflammation

The premise that atherosclerosis is an autoimmune disease is further enhanced by the observation that lipoproteins that have undergone oxidative modification stimulate haemoadhesive molecules, immunoregulatory molecules, inflammatory mediators and T-lymphocytes. However, atherosclerosis can also be categorised as an inflammatory condition because the leukocyte migration is determined by the state of the endothelium. Given the multifactorial nature of atherosclerosis, Ross (2006) proposed that the pathogenesis behind atherosclerotic plaques arises from the combined activity of the immune and inflammatory systems. The migration of leukocytes occurs in response to accumulating and modified lipoproteins as well as part of the inflammatory response.

The presence of ICAM-1, P selectin and VCAM-1 enhances the recruitment of leukocytes to the plaque growing on the endothelial surface of the artery. These adhesion molecules are further promoted by the presence of inflammatory cytokines, such as IL-1 and TNF-α (Mach, 2001).

2.7.6.2 Role of smooth muscles

Cytokines and growth factors are also synthesised by macrophages recruited to digest the modified lipoproteins; these proinflammatory factors attract more macrophages to the lesion as well as SMC. Plaque formation is mediated by PDGF and FGF, which are products of IL-1 and TNF-α stimulation. In response to PDGF released by activated endothelial cells, SMC migrate from the media to the intima. The secretion of matrix metalloproteinases (MMP) and MMP9 in particular, are considered to be important in SMC successfully migrating and proliferation, as the MMP degrade the elasticity of the lamina in cerebral arteries and the abdominal aorta. SMC proliferation is enhanced by the endothelial secretion of lipoprotein lipase; through the activation of protein kinase-C and the lipase enzyme binding to SMC

proteoglycan, gene expression for contractile proteins is turned off and other genes are activated. The result is the SMC synthesis of extracellular matrix, which contributes to the development and stabilisation of the plaque. TGF-β promotes the production of collagen and in common with interferon-γ, inhibits SMC proliferation. These factors acting together generate fibro-fatty lesions (Gawaz *et al.* 2000).

Development of lesions also involves neovascularisation of the vasa vasorum of the adventitia. The hormone, leptin, which is a product of the Ob-R gene, promotes angiogenesis. Elevated concentrations of leptin in both the plaque and the vasa vasorum stimulate inflammatory neovascularisation as well as functionally upregulating vascular endothelial growth factor (VEGF). Angiogenic growth is stimulated by the endothelial Ob-R gene generating a growth signal that activates tyrosine kinase-dependent intracellular pathways. OxLDL also contributes to atherosclerosis by inducing VEGF in macrophages. The matrix, thrombus and SMC proliferations associated with primary lesions in atherosclerosis are also implicated in the re-narrowing of arteries following angioplasty and stents (Glover *et al.* 2000).

2.7.7 Risk factors for atherosclerosis

The biggest risk factors for PAD are diabetes and smoking, though advanced age, hyperlipidaemia and hypertension are other recognised risk factors (Lee *et al.* 2009).

2.7.7.1 Hypertension

One of the challenges of studying hypertension as a PAD risk factor is that it is hard to establish whether rises in blood pressure are consequential or causal to PAD. Compared to healthy controls, the mean blood pressure and prevalence of hypertension in hospitalised vascular cases is generally higher. Studies of intermittent claudication in the general population show the phenomenon is related to raised systolic and diastolic pressures (Bulpitt, 1991), though most

commonly it is associated with raised systolic pressure. Fowkes *et al.* (1992) notes that in the Edinburgh Artery Study, individuals with symptomatic and asymptomatic PAD exhibited higher systolic blood pressure compared to non-PAD participants. This finding also supports the Framingham study, which at a 26-year follow-up, found there was a three-fold increase in intermittent claudication in hypertensive patients (Kannel *et al.* 1985).

In a study by Hirst *et al.* (2001), compared to controls, patients with PAD presented a greater incidence of hypertension ($P<0.001$). The control patients were also treated for hypertension less frequently than were patients with PAD ($P<0.001$). The researchers noted that patients with CVD received more intensive hypertension treatment than those patients who had newly diagnosed or prior PAD only ($P<0.001$).

2.7.7.2 Smoking

The contribution that tobacco makes to PAD pathogenesis is well recognised. PAD patients can reduce their risk of mortality and disease progression by stopping smoking (Jonason *et al.* 1987). Compared to patients with CVD only, the popularity of smoking was greater in patients with prior PAD ($P<0.001$) (Hirst *et al.* 2001).

Smoking cigarettes is a prime risk factor for PAD; this was the conclusion in 1965 when more than 90% of vascular patients attending hospital had a history of being a smoker, either currently or recently (Lord, 1965). The estimation is that almost half of the cases of PAD is attributable to smoking (Fowkes, 1988), which indicates that it is a key risk factor. This is borne out by the reduction in IC that is associated with the cessation of smoking. The Edinburgh Artery Study found that the relative risk of IC in smokers was 3.7 whereas in those who had ceased smoking for less than 5 years the relative risk was 3.0 (Fowkes, 1992).

2.7.7.3 Diabetes

Diabetes mellitus is a recognized risk factor for critical limb ischaemia and is related to the occurrence of milder forms of PAD. This has been discussed in further detail in the subsequent sections.

2.7.7.4 Inflammation

Persistent low-grade inflammation has been linked to asymptomatic PAD. In these patients who went on to develop PAD within the next 5 years, the levels of C-reactive protein (CRP) were higher than the levels in age-matched controls, who remained asymptomatic. The risk to those in the highest quartile of baseline CRP was more than double that of those in the lowest quartile (Ridker *et al.* 2001).

2.7.8 Investigation for detection of vascular insufficiency

2.7.8.1 Physical examination and history

The evidence indicates that the presence or absence of symptoms is unrelated to the progression of PAD. Hirsch *et al.* (2001) suggest that the presence or absence of intermittent claudication however, determines whether there is local deterioration and progression to critical limb ischaemia (CLI).

Intermittent claudication is the most common PAD symptom, which presents as pain, ache or cramp in the buttocks, thighs or calves when walking and resolves at rest. However, in extreme PAD, pain can occur at rest and can also include gangrene and loss of tissue. CLI describes these severe, limb-threatening presentations of PAD.

Classic claudication was present in 8.7% of the PAD-only group in Hirst *et al.*'s study (2001), though it was less prevalent in patients newly diagnosed with PAD compared to those with

prior PAD (5.5% c.f. 12.6%; P<0.001); it was appreciably lower in patients without atherosclerosis (1.7%; P <0.001). Using duplex scanning, the Edinburgh Artery Study found blockages in a lower limb artery in one third of asymptomatic PAD patients (Fowkes *et al.* 1991). In these asymptomatic patients, diagnosis was most likely to be determined through ABI measurement, as typically clinicians use claudication history to establish a PAD diagnosis, thereby missing between 85–90% of PAD diagnoses (Newman *et al.* 1997).

An initial clinical assessment of PAD typically includes a history and physical examination. Using intermittent claudication to identify PAD can be useful, but as indicated, it grossly underestimates PAD's actual prevalence. Examinations that use palpable pedal pulses, may eliminate diagnoses with a negative predictive value of more than 90%; on the other hand, using pulse abnormalities, such as absent or diminished beats, overestimates the prevalence of PAD. These results indicate the need to use objective measures to determine PAD in patients. Ankle brachial index (ABI) is the main non-invasive PAD screening test.

2.7.8.2 Investigations

2.7.8.2.1 Bedside investigation

2.7.8.2.1.1 Ankle Brachial Pressure Index

In the general population, the prevalence of asymptomatic PAD in lower limbs can only be estimated with non-invasive measurements; but where PAD is suspected, ABI can be administered at the bedside to identify PAD. Using a 10–12 cm sphygmomanometer with the cuff located just above the ankle to measure ankle artery pressures and a Doppler instrument to measure the systolic pressure of the posterior tibial and dorsalis pedis arteries in each leg. The ABI is derived from normalising these pressures to that of the higher brachial pressure of either arm. The leg with the lower ABI is usually defined as the index leg.

A haemodynamic definition of PAD is often defined by a resting ABI of ≤ 0.90, which is attributed to haemodynamically significant arterial stenosis. This method that uses an ABI ≤ 0.90 reliably identifies 95% of symptomatic arteriogram-positive PAD individuals and almost 100% of healthy controls (Norgren *et al.* 2007).

2.7.8.2.2 Imaging tests

Not all lesions are suitable for revascularisation, but imaging is an effective means of identifying those arterial lesions that may be suitable by using open surgery or endovascular techniques. Revascularisation should be determined based upon the extent of a patient's walking ability, and the functional limitations imposed by the state of the vasculature. This includes claudication distance and the extent to which it affects a patient's day-to-day life, their independence and self-care capacity. So long as there are no contraindications that would preclude endovascular or surgical intervention, imaging and revascularisation are essential for CLI cases.

At present, imagining options include angiography, computed tomographic angiography (CTA), duplex ultrasound and magnetic resonance angiography (MRA). When selecting the imaging modality, contraindications and potential side effects need to be considered.

2.7.8.2.2.1 Angiography

Although angiography is expensive and is not risk free, it is the 'gold standard' imaging test. Approximately 0.1% of patients who undergo this test have a severe reaction to the contrast medium. The technique also carries a 0.16% risk of mortality. Atheroembolism, arterial dissection, access-site complications, including haematoma and renal failure arising from the contrast medium are included among the complications of angiography. Technological advancements have ameliorated several of these issues, such as using digital subtraction

angiography, intra-arterial pressure measurements across the stenosis (with and without a vasodilator) non-ionic contrast agents, and superior image projection and storage. Also, instead of traditional contrast media, magnetic resonance contrast agents such as gadolinium can be used. In patients who are vulnerable to renal impairment, partial studies that reduce the amount of contrast medium used and amount of time required, are more suitable than imaging the entire infrarenal arterial tree, as the risks are lowered. Nonetheless, full angiography that enables all arteries between the kidneys and feet to be viewed using digital subtraction angiography (DSA) is preferable for most instances.

2.7.8.2.2.2 Duplex ultrasound

An alternative to angiography is colour-assisted duplex imaging. This technology is much cheaper and safer than angioplasty; a skilled practitioner can extract key anatomic data including functional information, such as velocity gradients across stenoses. Visualisation of the lower extremity arterial tree can be achieved and include an accurate assessment of the extent and severity of lesions. A disadvantage of the technique is that it can take longer to conduct, and the results are influenced by the practitioner's skill.

2.7.8.2.2.3 Multidetector computed tomography angiography

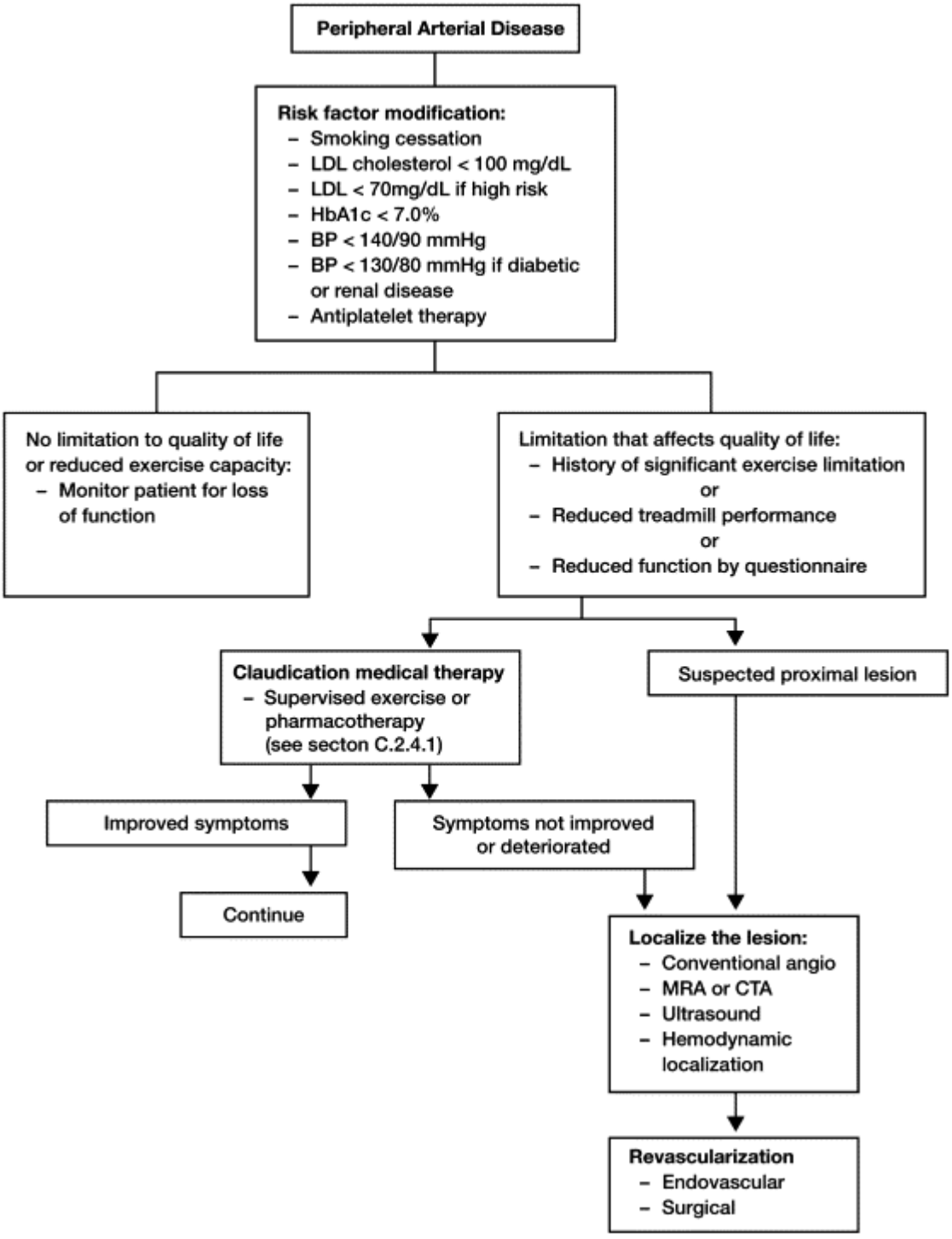
The level of morbidity and costs are considerably reduced with duplex scanning compared to other non-invasive techniques. CTA and MRA provide non-invasive imaging capable of evaluating the state of the lesions prior to invasive angioplasty; they can be applied to numerous situations. Frequently, PAD is being diagnosed using multi-detector computed tomography angiography (MDCTA), which is also used to determine appropriate treatment options. Familiarity with CT technology and the ease of using fast MDCTA multi-slice systems in the community have promoted the popularity of this technology.

2.7.8.2.2.4 Magnetic resonance angiography

MRA is the preferred choice of technology to diagnose and plan treatment of PAD patients in many health centres. In part, this reflects the safety of the technology, as well as its ability to generate high-resolution 3D images. MRA can successfully image the whole abdomen, lower extremities and pelvis in one session. 3D images have the advantage of being rotated across an infinite number of planes, giving full visual perspectives. The technique is appropriate for evaluating lesions for their suitability for endovascular intervention. Using MRA prior to a procedure may reduce radiation exposure and the use of iodinated contrast media.

2.7.9 Management of Vascular insufficiency

Figure 5: Overall treatment strategy for peripheral arterial disease



BP – blood pressure; HbA1c – hemoglobin A1c; LDL – low density lipoprotein; MRA – magnetic resonance angiography; CTA – computed tomographic angiography. Reproduced with permission from Hiatt WR. *N Engl J Med* 2001; 344:1608–1621.

To manage the disease state of a PAD patient, treatment needs to take into account the epidemiology and natural history of the disease as well as systemic disease risk factors that can be modified and those that adversely influence limb circulation.

2.7.9.1 Medical management

Claudication, which is reversible muscle ischaemia during walking, manifests as cramp or pain in the muscles involved in walking. The effect of the symptoms can be significant, greatly curtailing the amount of walking and exercise a sufferer can undertake. Consequently, patients with claudication are to varying extents, disabled and treatments aim to ameliorate symptoms and promote functional walking/exercising ability. Structured exercise ought to be the initial means to treat the symptoms, though some patients may be directed to pharmacological interventions to reduce claudication during exercise. For example, antiplatelet therapy and modification of risk factors may be required to minimise the risk of cardiovascular events and promote survival). Escalation to limb revascularisation is likely to be considered if structured exercise and/or pharmacology fail to make the desired improvements.

2.7.9.1.1 Antiplatelets

Hirst *et al.* (2001) report that 34% of the control group received antiplatelet drug therapy, which is lower than the number of patients who had already been diagnosed with atherosclerotic syndromes ($P<0.001$). In PAD-only patients, those who were recently diagnosed received less antiplatelet therapy than those who had a longer-standing diagnosis ($P< 0.001$). Antiplatelet therapy was more likely to be administered to CVD-only patients than those with PAD-only

(P<0.001).

2.7.9.1.2 Statins

Statins play an important role in managing patients with atherosclerosis. However, as majority of patient with PAD are underdiagnosed, they are not prescribed commonly. According to a cross sectional study by Hirst *et al*. (2001), the levels of LDL cholesterol had been obtained from only 63% of newly diagnosed and 65% of previously diagnosed PAD-only patients; in contrast, the LDL levels were identified in 73% of CVD-only patients (P<0.01 for prior PAD only c.f. CVD-only). The difference in the prevalence of hyperlipidaemia in the PAD and control groups was statistically significant (P<0.001). Those patients who had recently been diagnosed with PAD received less intense hyperlipidaemia treatment, which was comparable to the control group, compared to those who had been diagnosed previously (P < 0.006). Of all groups, the CVD-only patients received the most intensive therapy for hyperlipidaemia (P<0.001).

2.7.9.2 Surgical management

Acute limb ischaemia is a surgical emergency. For patients who have a profoundly ischaemic limb and have undergone severe loss of motor and sensory capability in a short period of time, immediate revascularisation may be indicated. However, the extent of success is largely determined by the speed at which revascularisation is performed after onset. The window is likely to be measurable in hours, with marked recovery possible if the procedure is completed very quickly. But as the window closes, significant neuromuscular damage is almost certain. Whether revascularisation is endovascular or open surgery largely depends on the location of the blockage. In the past, surgery was the treatment of choice for urgent treatment, but with as endovascular management has progressed and greater understanding about the importance of

circulation significantly promoting patency, where endovascular services are available, the time window for immediate revascularisation has been widened (TASC, 2000).

2.7.9.2.1 Angioplasty

The success of percutaneous transluminal angioplasty (PTA), as determined by patency, tends to be highest in the common iliac artery, with literature reporting technical and clinical successes exceeding 90%. PTA resolves almost 100% of focal iliac lesions. The technical success rate of revascularisation of long segment iliac occlusions is 80%–85% with or without additional fibrinolysis. The technical success of revascularisation has benefitted from device developments aimed at treating entire blockages. PTA is progressively less effective for lesions in distal arteries (Saket *et al.* 2004).

Tetteroo *et al.* (1998) conducted a prospective, randomised, multicentre study into the outcome of primary stents compared to PTA with provisional stenting. They found that the intervention rate of 7% of the latter technique was comparable to the 4% of primary stents (not significant). A similar pattern was observed at 5 years (mean 5.6 years ± 1.3) after treatment, with 82% of PTA and provisional stenting and 80% of primary stents not requiring further revascularisation of the iliac artery segments (Klein *et al.* 2004).

2.7.9.2.3 Bypass

Where there is diffuse disease throughout the aortoiliac segment, bilateral bypass surgery from the infra-renal abdominal aorta to both femoral arteries is usually recommended. The interest in endarterectomy has recently undergone a bit of a renaissance, though its technical challenges make it less commonly practised than bypass grafts. In a report by Rothwell *et al.* (2004) exploring primary patency rates 5-years after intervention, the researchers found a considerable variation (60–94%), which was attributed to the skill of the professional performing the

endarterectomy.

There is limited randomised trial data comparing bypass surgery and PTA for infrainguinal arterial obstructive disease. This can in part be accounted for by bypass surgery typically being performed in disease states with long lesions and CLI. In contrast, PTA is used more often where the disease is less extensive, obstructions are short and IC. One prospective, randomised, multicentre trial included 262 men with obstructions in the iliac, femoral or popliteal artery. Patients were randomly allocated to bypass surgery or PTA intervention; a 4-year median follow-up that evaluated patency, limb salvage and survival found no significant difference between interventions. Primary patency a further year on was 43% in 56 PTA patients, and 82% in bypass surgery patients. This indicates that surgery is superior to PTA where there are long superficial femoral artery (SFA) stenosis or blockages. Adams *et al.* (2005) found in their randomised study of 452 patients that there was no survival difference at 6 months in patients who had not undergone amputation. However, surgery is a more expensive option.

2.8 Diabetes

2.8.1 Introduction

Diabetes mellitus is defined as a group of metabolic diseases characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. In the long-term, various organs including eyes, kidneys, nerves, heart, and blood vessels become damaged because of chronic hyperglycaemia associated with diabetes mellitus (American Diabetes Association, 2013). According to Wild *et al.* (2004) in 2000, the number of people globally suffering from diabetes mellitus was 171 million; by 2030, this figure is expected to rise to 366 million. The disease is responsible for increased morbidity and mortality because of microvascular complications, as well as a higher risk of macrovascular complications, including ischaemic

heart disease, stroke and peripheral vascular disease, and thereby resulting in reduced quality of life (World Health Organisation, 2011).

During the period 2006-2011, there has been a 25% increase in the number of people that were diagnosed with diabetes mellitus in the United Kingdom, rising from 1.9 million to 2.5 million (Quality Outcome Framework Prevalence Data, 2012). Furthermore, the number of people with undiagnosed diabetes mellitus is thought to be around 850,000. Concomitantly, diabetes mellitus-related complications are also on the increase. At present, amputations, strokes, blindness and end-stage kidney failure occur primarily due to diabetes mellitus. Based on the existing trends, it is expected that the number of people in the United Kingdom suffering from diabetes mellitus will reach 5 million by 2025. Type 2 diabetes mellitus is the most common form of the disease, with 90% prevalence, while type 1 diabetes mellitus occurs in a proportion of 10% (Diabetes UK, 2011).

Hex *et al.* (2012) reported that the treatment of diabetes mellitus takes up approximately 10% of the budget of the National Health Service (NHS). A report by Diabetes UK (2011), confirmed this and reported that in 2011, the treatment of diabetes mellitus accounted for 10% of the NHS budget, amounting to £10 billion. A proportion of 80% of these costs were associated with the treatment of preventable complications, such as diabetic foot ulcers. Together with indirect costs, which pertain to mortality rates, sickness, possible reduction in productivity among people in employment, and informal care, these direct costs amounted to £23.7 billion for 2010-2011 and the figure is predicted to increase to £39.8 billion by 2035-2036 (Hex *et al.* 2012).

2.8.2 Vascular complications of diabetes mellitus

Chronic vascular complications may be further classified into macrovascular and

microvascular complications). Vascular complications of diabetes mellitus shorten life expectancy on average by 16 to 20 years in type 1 diabetes mellitus patients and by 4 to 6 years in those with type 2 diabetes mellitus (Trento *et al.* 2013).

One of the commonest complication which results from diabetic neuropathy and macrovascular complications of diabetes mellitus like peripheral vascular disease is diabetic foot disease. A diabetic foot ulcer is in most cases the first sign of lower limb amputation.

2.8.3 Epidemiology of vascular complications of diabetes mellitus affecting amputation

The available data for the global prevalence rate of amputation in numerous countries is poor, though the 5-year mortality rate is noted to be very high (up to78%) (Aleccia, 2010). In 2005, a number of about 664,000 of successful cases of major amputation and nearly a million cases of minor amputation were recorded globally (Ziegler-Graham *et al.* 2008). Moxey *et al.* (2010) further reported that, from 2003-2008, five out of every 100,000 individuals had a major amputation surgery, although considerable regional variation was noted. Vascular insufficiency and severe trauma account for 54% and 45%, respectively, of the approximately 5,000 amputations performed each year in the UK (Aleccia, 2010). Furthermore, foot ulcers have been estimated to be developed by about 61,000 individuals who account for around 2.5% of the population of diabetics in the UK. According to the statistics of the National Diabetes Audit (2010), in 2009-2010, 7 and 13 out of every 10,000 individuals had major and minor amputation, respectively, of a lower limb. Another study noted that, during the period 2007-2010, for every 10,000 diabetics there were 25 major amputations of lower limb (Holman *et al.* 2012).

2.8.4 Cost implications of diabetic mellitus related amputations

Diabetic foot disease often leads to serious long-term complications, putting significant socio-

economic pressure on resources and health care. The National Health Service is under enormous financial strain as a result of diabetic foot complications, reflected in greater outpatient costs, bed occupancy, and extended hospitalisation. The cost of diabetic foot care to the UK National Health Service during 2010-2011 was £639-662 million. A proportion of 10% of the NHS budget is taken up by diabetes and diabetes-related complications account for 80% of the total cost. Li *et al.* (2010) estimated that, taking into consideration modifications in demography and the increasing rate of obesity, in the coming two decades, diabetes mellitus will come to represent 17% of the whole NHS budget, if the current cost of diabetes treatment remains unchanged (Kerr *et al.* 2012). These figures do not take account of the indirect costs to patients such as the effect on physical, psychological and social wellbeing (Singh *et al.* 2005). Social care will also require additional funding because individuals who undergo amputations require assistance in daily activities and in caring for themselves. Hence, diabetes mellitus will place an even greater strain on the health and social care system. What is more, diabetics also have significantly lower work productivity due to poor health, the cost of which has been approximated at around £9 million, though further research is needed with to corroborate this (Hex *et al.* 2012). The International Diabetes Federation has estimated that around 50% of direct healthcare costs are due to loss of work productivity in the United States (Yang *et al.* 2012).

2.8.5 Macrovascular complications of diabetes mellitus and their role in amputation

Diabetes mellitus is often accompanied by macrovascular complications. The morbidity and mortality rates among patients with diabetes mellitus are significantly increased by vascular disease, which, together with diabetes mellitus, is responsible for the greatest number of deaths on a global level (Nuzum and Merz, 2009).

2.8.5.1 Peripheral arterial disease and amputation

As explained by Ross (1986), peripheral arterial disease is often associated with diabetes mellitus and is characterised by progressive decrease in blood flow to at least one extremity because of atherosclerosis. This has been discussed in further detail in the previous sections.

2.8.5.2 Hypertension and amputation

A diagnosis of hypertension is established when the average of at least two measurements of diastolic blood pressure (BP) on two or more visits is equal to or higher than 90 mmHg or when the average of several measurements of systolic blood pressure on at least two visits is constantly equal to or higher than 140 mmHg. The correlation between lower limb amputation and hypertension as well as between lower limb amputation and peripheral vascular disease led Lehto *et al.* (1996) to conclude that vascular aetiology is the cause of lower extremity amputation in numerous cases. Isolated systolic hypertension is identified when the systolic and diastolic BPs are respectively equal to or higher than 140 mmHg and lower than 90 mmHg. High blood pressure has also been implicated as a risk factor for lower limb amputation Hamalainen *et al.* (1999). According to Moss *at el.* (1999) who evaluated the risk factors for lower limb amputation looking at a cumulative 14-year incidence noted that lower limb amputation was related to higher diastolic blood pressure (OR for 10 mmHg 1.58 [1.20-2.07]) in the multivariate analysis.

2.8.6 Microvascular complications and their role in amputation

2.8.6.1 Diabetic retinopathy and amputation

Diabetic retinopathy is a part of the microvascular complications of diabetes mellitus. It has been noted to be a marker for atherosclerosis and a risk factor for lower limb amputation in

patients with diabetes mellitus. The condition of the vascular system can be inferred from the condition of the retina and the changes in the retinal arterioles may signal a deterioration occurring elsewhere (Wong *et al.* 2001). Retinal arterioles, which can be observed without invasive procedures, are similar to the cerebral and coronary circulations in terms of their anatomy and physiology (Singerman *et al.* 1991). According to some studies, apart from hypertension, inflammation and endothelial dysfunction, the narrowing of retinal arterioles may also be a marker of coronary heart disease (Klein *et al.* 2002). The correlation between changes in retinal arterioles and the rate of lower extremity amputation was investigated by Moss *et al.* (2003) based on a sample of 996 individuals who developed diabetes mellitus at a younger age, with follow-up over a period of two decades. Their findings suggested that, compared to patients without generalised arteriolar narrowing, those with it were more likely to have a lower limb amputated (15.7% vs 5.7%; OR 3.08; 95% CI 1.60-4.68); likewise, the risk of lower limb amputation was also high among patients with focal narrowing (33.1% vs 6.8%; OR 5.59; 95% CI 3.27-9.54).

2.8.6.2 Diabetic nephropathy and amputation

Diabetic nephropathy is a common complication of diabetes mellitus. About half of patients with type 1 diabetes mellitus with overt nephropathy develop End Stage Renal Disease within a decade, while over 75% develop it within two decades if they do not undergo treatment (Chen *et al.* 2004). In comparison to individuals with type 1 diabetes mellitus, a larger number of individuals with type 2 diabetes mellitus develop microalbuminuria or overt nephropathy at the time of or immediately after being diagnosed with diabetes mellitus. As noted by Waanders *et al.* (2013), the reason for this may be that the disease was present long before it was diagnosed. Within a decade, 20-40% of patients with diabetes mellitus related microalbuminuria develop overt nephropathy, of which 20% develop End Stage Renal Disease within two decades (Amin

et al. 2013). Worsening renal function due to diabetic nephropathy is a well-known factor for poor healing in patients with PAD resulting in higher risk of an amputation.

2.8.6.3 Diabetic peripheral neuropathy and amputation

Diabetic peripheral neuropathy is one of the most common complications of diabetes mellitus. According to Aszmann *et al.* (2004), the prevalence of peripheral neuropathy is more than 50% in those who have been diabetic for 20 years. A recent definition of diabetic peripheral neuropathy put forth by Vinik *et al.* (2013) refers to it as a symmetric, length-dependent sensorimotor peripheral neuropathy caused by metabolic and microvascular abnormalities arising from chronic hyperglycaemia exposure due to diabetes mellitus and cardiovascular risk covariates. Both types of diabetes mellitus, as well as different forms of acquired diabetes mellitus, are associated with diabetic peripheral neuropathy (Dyck *et al.* 1993). Foot ulceration is the condition most commonly related to somatic peripheral neuropathy, often leading to gangrene and limb amputation. The risk of amputation is increased by peripheral neuropathy 1.7-fold, 12-fold in the case of deformity, which is caused by peripheral neuropathy and 36-fold if the patient has a history of earlier ulceration (Vinik *et al.* 2013).

Independent from large-vessel disease, sensory loss may result in an ulcer development and sometimes amputation as well (Akbari *et al.* 1998). Ulcer development has an annual incidence rate of 2.5%, with one in six diabetics experiencing it at some point in their life (Frykberg *et al.* 2006).

2.8.7 Pathophysiology of vascular complication of diabetes mellitus related to lower limb amputation

There are a lot of factors that play a role which result in a lower limb amputation, diabetes mellitus being the most common. As indicated by Chaturvedi *et al.* (2001), in more than 50%

of all cases, leg amputation is carried out as a direct consequence of diabetes mellitus and its complications. Furthermore, in conjunction with peripheral neuropathy, diabetes mellitus is responsible for the occurrence of foot ulcers; with approximately 3% of patients with diabetes mellitus developing foot ulcers annually. Foot ulcers which do not heal determine leg amputations in 85% of cases of individuals suffering from diabetes mellitus (Boulton *et al.* 2004). The ulcers generally develop in areas under extreme pressure, such as the distal ends of the first and fifth metatarsals, the calcaneum, and other areas which are prone to recurrent trauma like the extremities of the phalanges (Ledermann *et al.* 2002).

Peripheral neuropathy and ischaemia represent the two major risk factors for foot ulcerations and subsequent lower limb amputation. Studies have shown that 30% to 50% of patients with type 1 as well as type 2 diabetes mellitus are likely to develop peripheral neuropathy and ischaemia (Bowering, 2001).

2.8.7.1 Role of peripheral arterial disease in lower limb amputation

PAD is one of the important causes of lower limb amputation. This has been discussed in the earlier section.

2.8.7.2 Role of hyperglycaemia in lower limb amputation

Individuals with diabetic foot disease are more likely to undergo amputation if diabetes mellitus is inadequately managed which leads to vascular and neuropathic complications. Brownlee (2005) reported that amputation is directly correlated with high glycaemic levels.

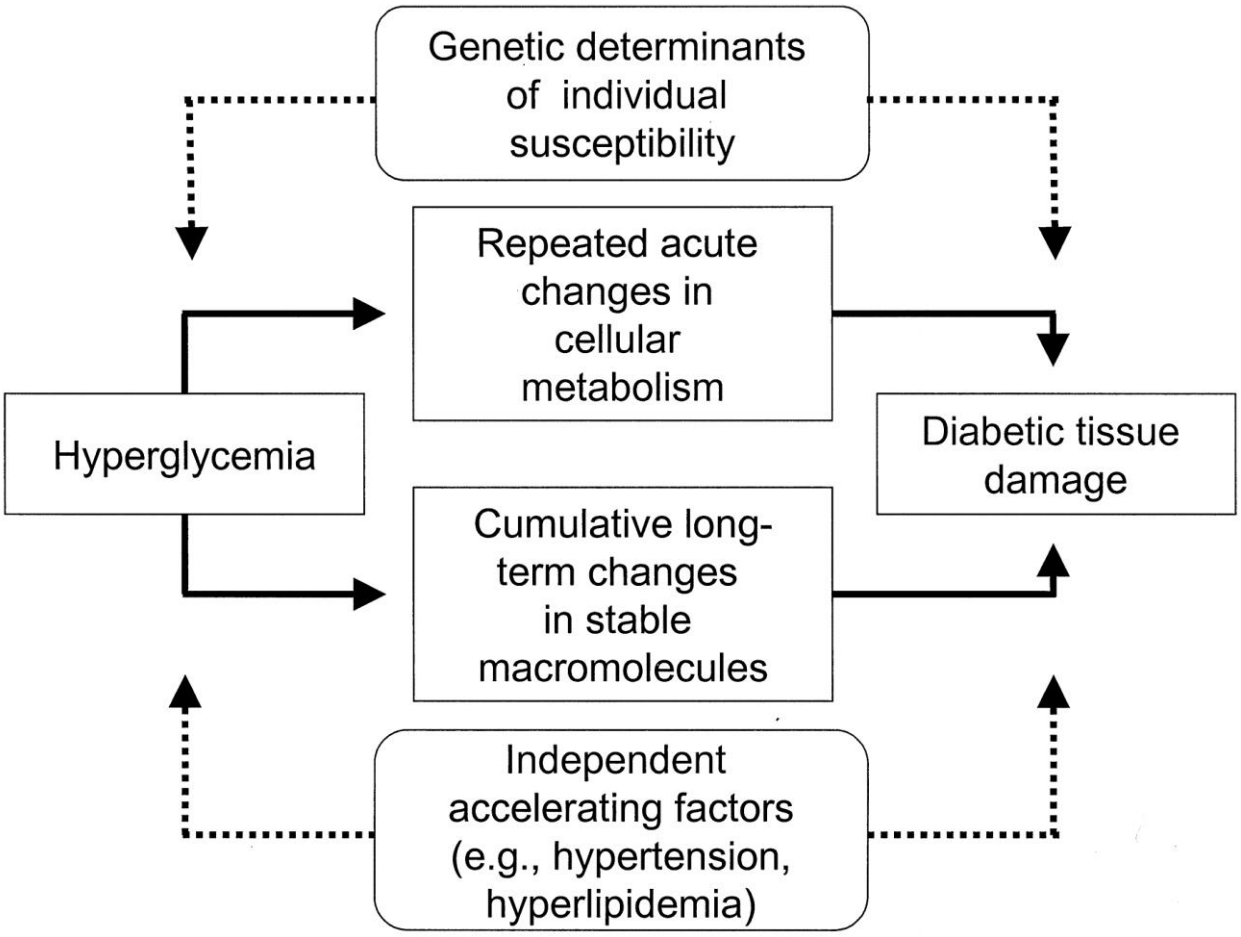
Parillo *et al.* (2004) highlighted that atherosclerosis, cerebrovascular disease, and peripheral vascular disease all have a greater probability of development in the case of individuals with diabetes mellitus. Diabetes mellitus disrupts normal nutrient metabolism and leads to an

increase in inflammatory mediators, thus interfering with wound healing and increasing the risk of infection and foot ulcers. The most prevalent foot injury that may cause a lower limb amputation is diabetic lower limb ischaemia due to arterial blockage (Armstrong *et al.* 1998).

Chronic hyperglycaemia is accompanied by a number of microvascular complications, including peripheral neuropathy and can undermine endothelial permeability, which can degenerate into endothelial dysfunction (Dang *et al.* 2005). Figure 6 and 7 illustrate the process which despite the high concentrations of plasma glucose present in all cells affected by diabetes mellitus, only the cell types which cannot control the transport of glucose into the cells, such as endothelial cells, are affected by hyperglycaemic disruption, causing intracellular hyperglycaemia (Brownlee, 2005). It also explains that there is genetic susceptibility as well as environmental factors at play which contribute to glucose related cell damage in patients with diabetes mellitus.

Figure 6: General features of hyperglycemia-induced tissue damage

Source: Brownlee M. 2005. Diabetes; 54:16 15-1625



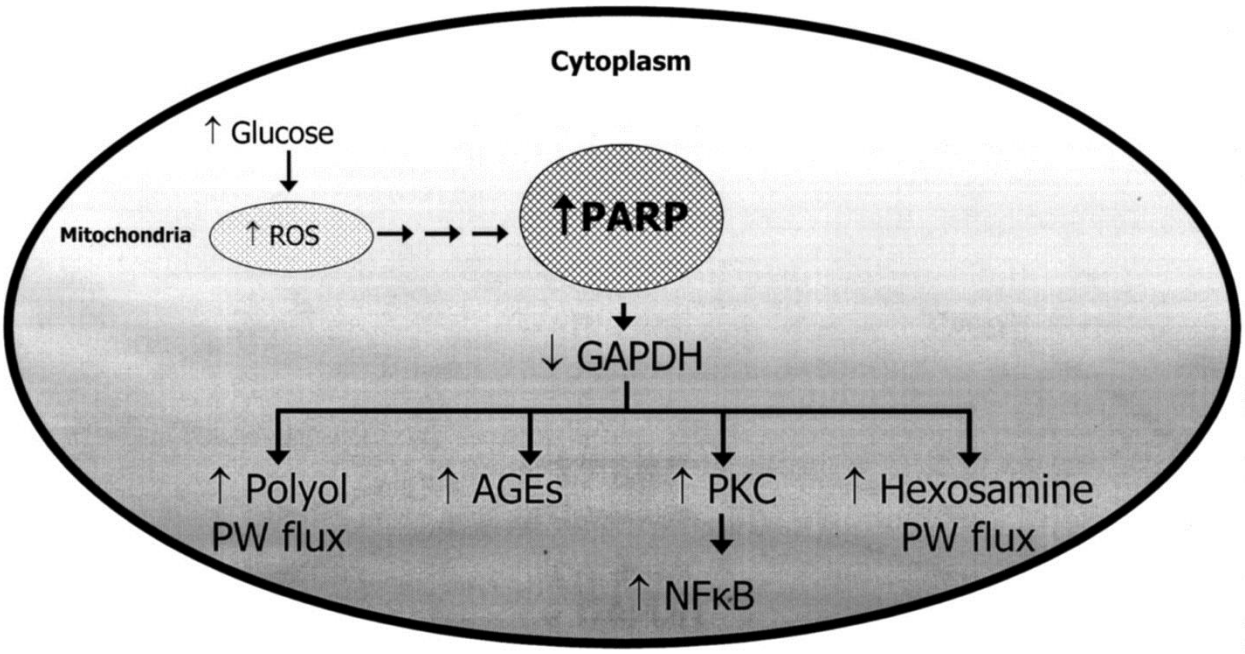
As noted by Brownlee (2005), in the initial phases of diabetes mellitus, intracellular hyperglycaemia manifests as increased circulation, vascular permeability and intra-capillary pressure. These complications are caused by the reduced function of vasodilators, such as nitric oxide and the accelerated function of vasoconstrictors, such as angiotensin II and endothelin-1 (Schmieder *et al.* 2009). As a result, there is an increased drainage of capillaries in certain

organs. In a study on diabetic animals, Brownlee (2001) observed that there was an over-expression of the Connective Tissue Growth Factor (CTGF), implying that it contributes to the development of microvascular and macrovascular diabetic complications.

Hyperglycaemia also results in mitochondrial Reactive Oxygen Species generation (mROS) (Brownlee, 2005), producing oxidative stress through a number of mediums, such as diacylglycerol/protein kinase C (DAG/PKC) and hexosamine that subsequently cause endothelial dysfunction and microvascular complications, including peripheral neuropathy, thereby leading to poor healing (Zhang and Gutterman, 2007). The Nuclear Factor-kappa B (NF-kappa B) family which comprises DNA-binding protein factors that are required for the transcription of most proinflammatory molecules. Various studies performed in a variety of cell and animal based experimental systems suggested that Nuclear Factor-kappa B activation was a key event early in the pathobiology of diabetes mellitus (Patel *et al.* 2009). Studies including Lupachyk *et al.* (2011) have highlighted the important role of poly (ADP-ribose) polymerase (PARP) activation in systemic oxidative stress in diabetic peripheral neuropathy.

Figure 7: The unifying mechanism of hyperglycemia-induced cellular damage

Source: Brownlee M 2005. Diabetes; 54:1615-1625



Abbreviations: ROS- Reactive Oxygen Species; DAG/PKC- diacylglycerol/protein kinase C; AGEs- Advanced glycation end products; GAPDH- glyceraldehyde-3-phosphate dehydrogenase; PARP- poly(ADP-ribose) polymerase; NFkB- Nuclear Factor kB

The wound healing process is frequently delayed in patients with diabetes mellitus. Endothelial progenitor cells (EPCs) which are produced by the bone marrow, have an important role in the creation of blood vessels and wound healing. In their study on diabetic mice, Velazquez *et al.* (2007) noted in mouse models that diabetes mellitus is related to a reduction in the amount of Endothelial progenitor cells in the blood flow and in the area surrounding a wound. According to the researchers, the wound healing process in patients with diabetes mellitus is hampered by

the impaired endothelial Nitric Oxide Synthetase (eNOS) activation, which stimulates nitric oxide production, and by the reduced concentration of chemokine Stromal cells Derived Factor 1 alpha (SDF-1alpha). Disruptions in the production or function of growth factors, angiogenic reaction, macrophages, collagen accumulation, epidermal barrier, amount of granulation tissue, keratinocytes and fibroblasts, could also contribute to inefficient wound healing in patients with diabetes mellitus (Maruyama *et al.* 2007)

2.8.7.3 Endothelial dysfunction and lower limb amputation

As mentioned in the previous two sections, patients with diabetes mellitus have hyperglycaemia related endothelial damage. This is compounded by the deceased vascularity due to pre-existing arterial insufficiency and infection. This results in further cell damage at the cellular level. The pathogenesis of diabetic vascular complications frequently arises in response to endothelial dysfunction, which is associated with the upregulation of inflammatory mediators and increased expression of Cell Adhesion Molecules (CAMs) (Nyström *et al.* 2006). The over-expression of Cell Adhesion Molecules promotes leukocyte-endothelial interactions, which further stimulates the inflammation response leading to tissue damage. The levels of plasma soluble adhesion molecules have been found to be elevated in type 2 diabetes mellitus patients. Studies by Sármán *et al.* (1998), found that Endothelin-1, a potent vasoconstrictor with mitogenic capability, promotes proliferation of vascular smooth muscle cells, which contributes to the development of atherosclerosis.

The release of endothelins contributes to the endothelial dysfunction that is commonly found in patients with diabetes mellitus. Sánchez *et al.* (2001) found a correlation between patients with poor glycaemic control and higher plasma levels of Endothelin-1. Indeed, the levels were even greater in patients with diabetes mellitus suffering from vascular disease complications.

This may be modulated by pharmacological intervention, as Schneider *et al.* (2002) noted that diabetic patients taking Angiotensin Converting Enzyme (ACE) inhibitors exhibited lower Endothelin-1 levels than those not taking Angiotensin Converting Enzyme inhibitors. Various studies have found that plasma Endothelin-1 concentrations were abnormally high in patients with conditions associated with endothelial cell injury, as well as in those with hypertension (Hiramoto *et al.* 2009), congestive heart failure (Kinugawa *et al.* 2003), coronary artery disease (Sánchez *et al.* 2001), and uraemia (Deray *et al.* 1992).

In addition to Endothelin-1, Nitric Oxide (NO) is important in regulating the homeostasis of vascular tissue. Nitric Oxide is an endogenous vasodilator, synthesized by endothelial cells (Brownlee, 2005); it protects vascular tissue by countering the abnormal proliferation of vascular smooth muscle cells that occurs after vascular interventions, for example a bypass graft (Vural *et al.* 2001). Nitric Oxide has also been implicated in reducing monocyte adhesion and inhibiting platelet aggregation (Marin *et al.* 1997). An interesting observation was reported by Hattori *et al.* (1991) in which the release of Nitric Oxide and the response to it was reduced at the onset of diabetes mellitus. In summation, Endothelin-1 and Nitric Oxide are important mediators in maintaining vascular function.

C-reactive protein (CRP) is a marker of inflammation, which is elevated in patients with Diabetic Mellitus with peripheral arterial disease. The level of C-reactive protein correlates to the severity of the arterial disease. Endothelial cells manufacture various proteins, including Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Adhesion Molecule-1 (VCAM-1). According to Bevilacqua (1985) and Karaduman *et al.* (2006) their expressions increase after stimulation by proinflammatory cytokines and the tissue Intercellular Adhesion molecule-1 levels were positively correlated with blood glucose levels. Adhesive interactions between leukocytes and endothelial cells are involved in inflammatory or immunologic response

mechanisms. The adhesion molecules present on the surface of endothelial cells bind to the CD11a and CD11b/CD18 integrins on the surface of leukocytes; these integrins, which are abundant on polymorphonuclear leukocytes, are only expressed by White Blood Cells (Henderson *et al.* 2001). According to DiPiro *et al.* (1997) lymphocyte CD11a/CD18 play an important role in determining the response to infection influencing the type of immunity and the inflammatory response to infection. CD11b/CD18 expressed by neutrophils is key in binding the neutrophil to the surface of vascular endothelial cells (Diamond *et al.* 1991). Jaeschke *et al.* (1991) reported that the expression of CD11b/CD18 on neutrophils correlates with microvascular dysfunction and is elevated in patients with infections.

2.8.7.4 Role of hypertension in endothelial dysfunction and lower limb amputation

The association between hypertension and endothelial dysfunction is well recognized (Panza *et al.* 1995). Based upon data collected from the Framingham offspring cohort study, there is a positive correlation between the severity of hypertension and the extent of endothelial dysfunction (Benjamin *et al.* 2004). Several studies have drawn a link between the increases in systemic oxidative stress and vascular inflammation associated with hypertension (Harrison *et al.* 2009). Sources of oxidative stress associated with hypertension include mitochondria and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase (Widder *et al.* 2009). Studies by Vecchione *et al.* (2009) performed in animals concluded that isolated carotid arteries from mice when exposed to increasing intraluminal pressure showed a concomitant decrease in endothelium-dependent vasodilation to acetylcholine, increased vascular superoxide production, and increased Nicotinamide Adenine Dinucleotide Phosphate oxidase activity. Hypertension induced Reactive Oxygen Species (ROS) derived from mitochondria and Nicotinamide Adenine Dinucleotide Phosphate oxidase contributes to endothelial dysfunction (Doughan *et al.* 2008).

2.8.7.5 Role of obesity in endothelial dysfunction and lower limb amputation

Obesity is a disorder of complex aetiology determined by genetic as well as environmental factors. As a consequence of the development of obesity, a large proportion of individuals develop the insulin resistance syndrome, which is characterized by several metabolic abnormalities such as hyperinsulinemia, diabetes mellitus, dyslipidemia and hypertension (Lobato *et al.* 2012).

In recent years, the significance of adipose tissue in regulating metabolism and inflammation, as well as adipokines (cell signaling proteins) has been recognized by clinicians and researchers (Lobato *et al.* 2011). In obese patients, inflamed adipose tissue is linked to poor endothelial function (Dantas *et al.* 2004). Adipose tissue inflammation studies have explored the influence of perivascular adipose tissue on vascular homeostasis in hypertension. In studies using hypertensive rats, contrary to normal rats, adipose tissue was found to be inadequate in suppressing phenylephrine-induced vasoconstriction (Serpillon *et al.* 2009). In addition, hypertensive, obese rats with perivascular inflammation demonstrated greater endothelial dysfunction compared to normal rats (Akamine *et al.* 2006). Together, the data indicated that inflamed adipose tissue and perivascular adipose tissue make a significant contribution to regulating vascular homeostasis at local and systemic levels.

2.8.7.6 Role of distal peripheral neuropathy and lower limb amputation

This has been discussed in the earlier section.

2.9 Predisposing factors of lower limb amputation

There are numerous risk factors for leg amputation in the case of patients with diabetes mellitus,

as indicated by different studies. In their study of 202 patients, Nather *et al.* (2008) specified a number of risk factors for amputation, including older age (over 60 years), ischaemic cardiac disease, nephropathy, peripheral vascular disease, sensory peripheral neuropathy, glycosylated haemoglobin, ankle brachial pressure index lower than 0.8, gangrene, infection and pathogen invasion, such as *methicillin-resistant Staphylococcus aureus*. According to Younes *et al.* (2004), the most common risk factors for foot ulceration, which most commonly leads to lower limb amputation are: diabetic peripheral neuropathy, anatomical deformity of foot, and peripheral vascular disease. Santos *et al.* (2006) also identified a combination of factors which heightens the likelihood of amputation, namely, older patients with diabetic foot infection, long-term diabetes mellitus, advanced lymphangitis, wounds in the calcaneum area and grade 5 injuries in Wagner's classification. Other risk factors are discussed in the following section.

2.9.1 Diabetes and lower limb amputation

Patients with diabetes mellitus account for 42% of the referrals made for prosthesis fitting. In the past eight years, there has been a substantial increase in the number of referrals for lower limb amputation, with diabetes mellitus being identified as the main cause (NASDAB, 2005). Among all the complications of diabetes mellitus, limb loss is perhaps the most distressing for the patient. Amputation has an extensive effect on the lifestyle of the patients, as well as on the healthcare budget (van Houtum *et al.* 2004). Dillingham *et al.* (2005), who looked at 12-month re-amputation and mortality rates in 3565 patients, concluded that diabetic amputees were younger by about seven years than the patients without diabetes mellitus (48 deaths in patients with diabetes mellitus with less than 75 years of age vs 24 in the non-diabetic group). In addition, diabetic amputees were generally of male sex, suffered from co-morbidities, and the first amputation they had been subjected to, was carried out at a younger age than in the case of patients without diabetes mellitus. Furthermore, they also observed that the age at death of

the patients with diabetes mellitus who had undergone amputation was also lower than that of those without diabetes mellitus.

2.9.2 Peripheral vascular disease and lower limb amputation

Peripheral vascular disease develops in the peripheral arteries usually of the lower limbs (Hirsch *et al.* 2006) and is a common cause for lower limb amputation. This has been discussed in further detail in earlier sections.

2.9.3 Diabetes and peripheral vascular disease

The prevalence of peripheral vascular disease is around 10% in patients with diabetes mellitus, whereas 2.6% of people who do not suffer from diabetes mellitus develop peripheral vascular disease (Gregg *et al.* 2004). Peripheral vascular disease has been indicated by the United Kingdom Prospective Diabetes Study (UKPDS) to occur more frequently in patients with type 2 diabetes mellitus, in contrast to the non-diabetic population. It is considered a risk factor for foot ulceration and amputation in patients with diabetes mellitus (Adler *et al.* 1999; Boyko *et al.* 1999). Approximately 75-85% of lower extremity amputations are as a result of peripheral vascular disease, of which up to 42% have coexisting diabetes mellitus (Donohue *et al.* 2001). Peripheral vascular disease in patients with diabetes mellitus increases the risk for lower extremity amputation and mortality.

2.9.4 Diabetic peripheral neuropathy and lower limb amputation

Diabetic peripheral neuropathy plays a major role in lower limb amputation. This has been discussed in further detail in an earlier section.

2.9.5 Renal disease and lower limb amputation

Patients with End Stage Renal Disease often develop diabetic peripheral neuropathy and peripheral arterial disease, which are prominent causes of leg amputation, thereby making patients with End Stage Renal Disease more prone for an amputation (Papanas *et al.* 2007). Hill *et al.* (1996) noted that End Stage Renal Disease patients have a 25% chance of developing diabetic foot complications, in contrast to diabetic patients not undergoing renal replacement therapy, who only had a 10% chance. There is a strong relation between deranged kidney function and unsuccessful healing of amputated stumps, as well as between albuminuria and risk of amputation (Ghanassia *et al.* 2008).

As pointed out by various researchers (Wolf *et al.* 2009), there is an almost eight-fold increase in lower limb amputation among patients suffering from peripheral vascular disease and severe chronic kidney disease, than among healthy patients. In their studies, Nather *et al.* (2008) and Broumand (2007) have argued that in the case of patients with diabetes mellitus, nephropathy and haemodialysis increase the likelihood of leg amputation. Patients with End Stage Renal Disease who have had a minor amputation in order to preserve a limb are at a higher risk of having a major amputation in comparison to patients who do not have End Stage Renal Disease (Sheahan *et al.* 2005).

2.9.6 Past medical history of amputation as a risk factor for lower limb amputation

Individuals who have undergone amputations in the past are more likely to be subjected to the procedure again (Adler *et al.* 1999), particularly if the amputation level was not selected correctly, which can have a negative impact on the healing rate (Izumi *et al.* 2006). Diabetics subjected to primary digit amputation for sepsis are more predisposed to develop repeated infections, which can lead to additional amputations (Nehler *et al.* 1999). This has been corroborated by the results obtained by Dalla-Paola *et al.* (2003), who noted that 10% of

patients with diabetes mellitus with first digit amputations had been subjected to a further higher amputation after less than a year and a half of the original amputation. Similarly, Murdoch *et al.* (1997) observed that 51% of patients had undergone a higher amputation in the same leg less than a year after their initial first digit amputation. Sheahan *et al.* (2005) estimated that the first six months following an amputation represented a high-risk period for an additional ipsilateral amputation. About to re-amputations of the contralateral limb, 23-30% of them occurred after three years of the initial amputation and 51% after five years (Braddeley, 1965). Larsson *et al.* (1998) indicated that the probability rates of re-amputation were 14%, 30% and 49% at one, three and five years, respectively. As major amputations are usually determined by Peripheral Arterial Disease which is progressive in nature in most cases, it follows that peripheral arterial disease extended to the healthy areas of the limbs, causing re-amputation of the opposite side to the initial amputation (Izumi *et al.* 2006).

2.9.7 Failed revascularization and lower limb amputation

Revascularisation techniques are used to prevent/delay lower limb amputations. Popliteal to plantar arch vein graft bypasses had a high success rate of about 80%, thus promoting the application of vascular reconstruction whenever possible as a method of avoiding amputation (Fichelle, 2011). However, in some cases, it has been possible to prevent limb loss in patients with diabetes mellitus by improving peripheral blood circulation in the affected tibial or peroneal arteries through distal revascularization surgical procedures (Verhelst *et al.* 1997). Nevertheless, as indicated by Shaehan *et al.* (2005), the rate of limb loss was considerably high among patients who had undergone vascular reconstruction prior to amputation. It has been described that critical limb ischaemia has a three-year limb loss rate of 40% with conservative therapy including revascularization techniques (Albers *et al.* 1992). In the majority of the cases, the three-year bypass rates of calf arteries varied from 40% for prosthetic bypasses to 85% for

saphenous bypasses (Vraux *et al.* 2006). However, there are many limiting factors to bypass grafts success including the occurrence of infection around the area targeted for intervention and failure of revascularization techniques itself prompting the patient to undergo a lower limb amputation.

2.9.8 Nutritional status and lower limb amputation

Adequate nutrition should be integrated into the injury treatment as it is vital for the proper healing of the wounds. Healing may be slowed down, or the wound can relapse and the whole wound treatment can be put in jeopardy if nutrition is inadequate (Vaneau *et al.* 2007). Improper nutrition can affect the generation of fibroblasts, damage neo-vascularisation, and reduce cellular and humoral immunity. The metabolic requirements in the healing process are high and patients with malnutrition are unable to cope with this (Harding *et al.* 2002). For an amputation to heal, the patient should have an acceptable nutritional status, including a serum albumin level of at least 2.5 g/dL (Pinzur *et al.* 2008).

2.9.9 Infection and lower limb amputation

Diabetics have a 25% chance of developing foot ulcers during their lifetime (Singh, 2005) and more than 50% of patients with diabetes mellitus develop infections (Lipsky, 2004). In particular, infections caused by diabetic foot ulcers sometimes determine amputation (Bowering, 2001). Infection can also develop as secondary to autonomic peripheral neuropathy resulting in the malfunctioning of the sweat glands; consequently, the skin becomes dry and cracked, enabling bacterial invasion (Clayton *et al.* 2009). However, amputation is only used as a last resort when the infected ulcers are life threatening or are resistant to any other types of treatment (Philbin, 2006).

As noted by Thomas-Ramoutar *et al.* (2010), osteomyelitis – the infection of the bone tissue –

is usually the final stage, leaving no other option except an amputation of the limb in the majority of cases (Lavery *et al.* 2006). Osteomyelitis is a significant risk factor for leg amputation as it has a high prevalence rate among patients with diabetic foot disease (Centres for Disease Control and Prevention, 2005). The strain generated by the high morbidity and mortality rates of leg injuries in patients with diabetes mellitus, as well as their high treatment cost, is further increased by the implications of amputations carried out as a result of osteomyelitis (Thomas-Ramoutar *et al.* 2010).

2.9.10 Leg ulcers and lower limb amputation

History of ulceration in the lower limb increases the risk of amputation (Adler *et al.* 1999). Newly formed ulcers can rapidly expand, increasing the risk of limb loss. It has been shown that there is a direct causal relationship between formation of diabetic ulcers and amputation in almost 85% of cases (Reiber *et al.* 1999). Deep ulceration with uncontrolled infection in patients with diabetes mellitus complicated by peripheral vascular disease generally results in a lower-limb amputation (Pino *et al.* 2011).

A high-grade leg ulcer of Wagner classification (grades 3) significantly multiplies the chances of a lower limb amputation (Sun *et al.* 2011). According to Boulton (2001), the Wagner grade is directly proportional to the probability of an amputation. However, several researchers have criticized the system, citing that it fails to take into account the influence of ischaemia or infection and instead puts too much emphasis on injury depth and tissue condition (Frykberg. 2002; Boulton *et al.* 2008).

2.9.10.1 Diabetic foot ulcer

2.9.10.2 Introduction

Diabetic foot ulcer is a widespread and debilitating complication of diabetes mellitus, which often results in limb loss. Moreover, it is also associated with a high mortality rate as well as recurrence of unhealed ulcers. In the United Kingdom, majority of patients with diabetes mellitus undergo amputation due to diabetic foot ulcers, which constitute the main reason for non-traumatic limb amputation. The procedure is usually carried out above the ankle or within the foot. As stressed by Reiber *et al.* (1999), left undiagnosed or untreated, diabetic foot problems lead to a higher morbidity and mortality.

2.9.10.3 Predisposing factors for diabetic foot ulcer

Diabetic foot ulcer is mainly brought about by the three major factors of vasculopathy, peripheral neuropathy and predisposition to infection, but additional risk factors also contribute to its development (Frykberg, 1991; Sanders *et al.* 2010). To successfully manage diabetic foot problems and amputation prevention, it is necessary to detect these risk factors early on.

Some of the risk factors for a diabetic foot ulcer include diabetic peripheral neuropathy, ischaemia due to PAD, duration of diabetes mellitus, control of diabetes mellitus and foot ulcer, past history of foot ulceration.

2.9.10.4 Natural history of diabetic foot

The treatment of diabetic foot ulcers requires knowledge of the natural history of the diabetic foot. Although all aspects of foot disease are considered, the central event is the development of the foot ulcer in stage 3, which calls for immediate and aggressive treatment. The management of diabetic foot lesions requires a multidisciplinary effort in order to effectively deal with the mechanical, wound, microbiological, vascular, metabolic and educational elements. The natural history of diabetic foot comprises five stages (Edmonds, 2008):

Stage 1 - Normal foot

Stage 2 - High risk foot

Stage 3 - Ulcerated foot

Stage 4 - Infected foot

Stage 5 - Necrotic foot

2.9.10.4.1 Normal foot

It is imperative for individuals with diabetes mellitus to undergo annual screening for the detection of risk factors for foot ulcer, such as peripheral neuropathy, ischaemia, deformity, callus and swelling. If these are present, then the foot is at risk, otherwise it is normal.

2.9.10.4.2 Diabetic foot at risk

According to NICE guidelines 2015, when examining the feet of a person with diabetes mellitus, the following risk factors should be looked out for: peripheral neuropathy (use a 10-g monofilament as part of a foot sensory examination), limb ischaemia, ulceration, callus, infection and/or inflammation, deformity and gangrene.

The NICE guideline (2015) classifies the foot in three categories based on the number of risk factors, namely;

- Low risk: no risk factors present except callus alone.
- Moderate risk: with one risk factor present.
- High risk: with more than one risk factor present or history of previous ulceration or previous amputation or on renal replacement therapy

The active foot problems that should be checked include ulceration, spreading infection, critical limb ischaemia, gangrene, possibility of acute Charcot arthropathy, or unaccountable increased foot warmth, redness and swelling accompanied or unaccompanied by pain.

2.9.10.4.3 Ulcerated diabetic foot

The development of diabetic foot ulcer is life changing for a diabetic patient because it signals an increase in disease severity and occurrence of comorbidities. The wound can worsen quickly if it is not treatment early and effectively, and could lead to an amputation of the affected limb (Kerr, 2012).

Two types of diabetic foot ulcers have been identified by Edmonds (2006), namely, neuropathic ulcers associated with neuropathic feet, and neuroischaemic ulcers occurring in feet with ischaemia often related to peripheral neuropathy. Ulcers secondary to ischaemia only occur in up to 15 % of cases (International Diabetes Federation Clinical Guidelines, 2012).

The neuropathic foot is characterised by warmth and good perfusion with palpable pulses, which reduces sweating, causing the skin to become dry and likely to crack. The plantar side of the foot, beneath the heads of the metatarsals, and the plantar side of the toes are the primary locations for the development of neuropathic ulcers. As noted by Frykberg *et al.* (2006), ulceration is mainly caused by repetitive gait mechanical forces, which lead to the formation of the major lesions preceding neuropathic foot ulcers, namely, calluses. If it reaches excessive thickness, the callus will put pressure on the underlying soft tissues, leading to ulceration.

The neuroischaemic foot lacks warmth and pulses, whereas the skin is thin, lustrous and hairless. The subcutaneous tissue becomes atrophic, while peripheral neuropathy may avert periodic claudication and rest pain. The foot edges are the main location of occurrence of neuroischaemic ulcers, particularly the medial surface of the first metatarsophalangeal joint

and over the lateral part of the fifth metatarsophalangeal joint (Edmonds, 2006). Furthermore, the tips of toes and underneath excessively thick toenails are also commonly noted areas of development of ulcers. Almost half of all the foot ulcers are ischaemic ulcers (Prompers *et al.* 2007).

2.9.10.4.4 Infected diabetic foot

A crucial stage of evaluation is the detection of infection in individuals suffering from diabetic foot ulcers, although this is far from being an easy task. The importance of this cannot be emphasised enough, enabling the treatment of a minor infection before it degenerates into a severe one that frequently requires amputation (Lipsky *et al.* 2012). According to the statistics gathered by Wu *et al.* (2007), infection develops in around 56% of cases of diabetic foot ulcers, while amputation is necessary in around 20% of cases of diabetes mellitus associated with foot infection. However, almost 50% of patients do not exhibit the common symptoms of infection and inflammation (e.g. redness, heat and swelling), because arterial insufficiency often occurs alongside peripheral neuropathy. Consequently, as suggested by Edmonds *et al.* (2004), less apparent, 'secondary' indicators of infection, such as friable granulation tissue, wound undermining, bad smells, and wound exudates, should be looked out for.

2.9.10.4.5 Necrotic diabetic foot

Developing because of infection, ischaemia or both, necrosis can have severe consequences and can lead to limb loss (Ricco *et al.* 2013). Necrosis is managed differently, according to whether it is wet or dry. In the neuropathic foot, necrosis starts off as wet and in most cases, it is caused by septic arteritis that accompanies soft tissue infection, a complication of a digital or metatarsal ulcer. A septic thrombus frequently blocks the arterial lumen. In the neuroischaemic foot, by contrast, necrosis can be either wet or dry (Lepäntalo *et al.* 2011). Dry

necrosis in the neuroischaemic foot occurs in association with a significant decrease in arterial perfusion (Edmonds *et al.* 2008). Septic arteritis, underpinned by large vessel disease in the lower extremity, is also a widely encountered cause of a black toe (Cooney *et al.* 2011).

2.9.10.5 Management of non-infected diabetic foot ulcer

2.9.10.5.1 Conservative management

2.9.10.5.2 Offloading

The development of ulcerations is promoted by minor trauma, including recurrent stress and footwear-applied pressure (Frykberg *et al.* 2000). As noted by Armstrong *et al.* (2001), peak plantar pressures are not as high in the rear foot and medial arch as they are in the forefoot. To increase treatment efficiency, the pressure that is applied to the wound, particularly in the forefoot, must be kept to a minimum. However, the pressure on the plantar foot surface can be increased by irregular biomechanics arising from restricted joint movement and/or structural foot deformity (Cavanagh *et al.* 2010). Wound healing may be retarded by even light pressure (Millington *et al.* 2000). In addition to deterring healing, pressure that is not relieved enhances the likelihood of complications. Known as the offloading "gold standard", Total Contact Casting (TCC) is the most efficient and widely used offloading method employed in neuropathic wound care (Armstrong *et al.* 2001).

2.9.10.6 Management of an infected foot ulcer

2.9.10.6.1 General management

Armstrong *et al.* (2004) have advocated that wound cleansing after surgical debridement of dead tissue is not only complementary to systemic antibiotics, but also minimises the risk of recurrent infection. However, the lack of adequate randomised control trials means that no

single solution has been agreed upon as being of utmost efficiency with surgeons having a free choice about irrigant selection.

2.9.10.6.2 Role of topical antimicrobials

Treating heightened wound burden with topical antimicrobial strategies has intensified since bacteria have become increasingly resistant to antibiotics (e.g. *Staphylococcus aureus* exhibits resistance to methicillin) or due to other complications (e.g. infection with *Clostridium difficile*) (Chadwick, 2013). As explained by Lipsky *et al.* (2009), the reason for this intensification is that resistance is not promoted by antimicrobial agents applied topically as they only offer high local concentrations, without permeating unbroken skin or deeper soft tissue.

2.9.10.6.3 Deep tissue infection

Broad-spectrum antimicrobial agents should be used to treat deep tissue infection (e.g. cellulitis, lymphangitis, septic arthritis, fasciitis) as soon as it is detected, and alternative antimicrobial agents should be used if treatment appears to be ineffective, according to microbiological results (Scottish Intercollegiate Guidelines Network, 2010). Lipsky *et al.* (2012) recommended parenteral administration of antibiotics for all serious and certain moderate infections, while improvement of infection permits switching to oral administration. In most cases 1–3 weeks of therapy is sufficient for soft tissue infections. There is no pre-defined duration of antibiotic therapy, as this is dictated by how severe the infection is and by treatment response (Richards *et al.* 2011).

2.9.10.7 Osteomyelitis

Osteomyelitis is the dreaded complication of diabetic foot ulcer and involves deep seated

infection affecting the underlying bone (Lipsky *et al.* 2006). Individuals suffering from moderate to severe diabetic foot infection often develop osteomyelitis. The diagnosis of this condition during the early phases is not easy to achieve, but its detection and adequate treatment is essential for the healing of the wound (Frykberg, 2002). Chronic, extensive and deep wounds are susceptible to infection from the underlying bone. Osteomyelitis is signalled by the existence of a 'sausage toe' or visible bone. Osteomyelitis can be clinically assessed by introducing a sterile, blunt metal probe into the ulcer to inspect the hard, gritty feel of the bone (Lozano *et al.* 2010). A high or low probability is respectively indicative of the presence or absence of osteomyelitis depending on the results of the probe-to-bone test.

2.9.10.8 Management of a necrotic diabetic foot ulcer

Wet necrosis in the neuropathic foot is managed in most cases through surgical debridement, since the arterial circulation is generally good (Heikkinen *et al.* 2007). With regards to the neuroischaemic foot, Tannenbaum *et al.* (1992) recommended the removal of wet necrosis when it occurs in conjunction with extensive sepsis, regardless of the existence of pus. If there is no immediate threat to the limb and necrosis is contained to one or two toes, intravenous antibiotics may be administered to achieve infection control, prior to urgent revascularisation associated with digital or ray amputation, which has good chances of healing. However, as Schaper (2011) points out, not all patients may be adequate candidates for revascularisation. Furthermore, Edmonds *et al.* (2008) propose that antibiotics should be used to transform wet necrosis into dry necrosis, which subsequently may auto-amputate.

2.10 Criteria for selection of the level of amputation

After taking the decision to perform the amputation procedure, an appropriate amputation level has to be established and the surgical techniques have to be verified for accuracy (Pino *et al.*

2011). It is of the utmost importance to establish the most suitable amputation level for each patient as it influences the functioning of the prosthetic limb. To ensure that the patient enjoys as independent a lifestyle as possible, the options of bypass surgery vs primary amputation have to be weighed carefully. Burgess and Matsen (1981) who are considered pioneers in amputation surgery stated that a higher level of amputation resulted in increased disability. According to Gottschalk (2002) patients with above knee amputation who have a high energy expenditure for walking and taking on a prosthesis is harder for them in comparison to a below knee amputee.

The selection of the level of amputation depends on a number of factors. Based on studies of the impact of different methods of incision on the results of leg amputations, Datta *et al.* (2001) and Tisi *et al.* (2004) added that several aspects have to be taken into consideration, including healing rate, possibility of recovery, aspects related to prosthetics, the desires of the patient, hospital discharge, as well as the extent of affected tissue in the leg to be amputated. In addition, the functioning of the knee and hip, as well as the existence of any joint prostheses, has to be taken into account. As noted by Gibson *et al.* (2001), the decision regarding the amputation level has to be a balance between stump healing and optimizing limb function.

Even though important advancements have been made in the field of vascular surgery, the prevalence of amputations carried out due to vascular insufficiency developed in patients with diabetes mellitus continues to grow and what is more, many re-amputations are still performed because the level of the first amputation was poorly selected. In their examination of 615 cases of leg amputations, Wutschert *et al.* (1997) observed a vast variation in the success rate of amputations, from 10% to 50% over the last two decades. Gu, (2004) analysed the scoring system created in accordance with the pre-surgery angiogram to determine the condition of the run-off vessel in 390 subjects and observed that the failure rate of trans-tibial amputations

exhibited a 10-50% variation, with a mean failure rate of 20%.

In order to ensure as complete a recovery, Malone *et al.* (1981) recommended a number of goals that any amputation treatment should strive to attain: (i) employ peripheral vascular reconstruction to ensure minimum limb loss; (ii) quantitative evaluation of the most suitable level of amputation; (iii) carry out the most distal type of amputation to enhance the healing process; (iv) restore functionality with a prosthetic limb; (v) minimize hospitalization without adversely affecting recovery; (vi) cost-efficient treatment; and (vii) multidisciplinary care input. The researchers proposed taking into account two essential aspects when deciding where the amputation level should be. The first aspect was carrying out the most distal amputation, given the circumstances. The second aspect was ensuring that the blood circulation in the amputation site is adequate to support the healing process. This would be achieved by carrying out the amputation at the most appropriate level. However, Moore (1974) argued that neither of these two considerations is feasible for a standard application. Reliance on the first consideration determined several unsuccessful healing processes which called for additional surgery, endangering the life of the patient. The level of the last amputation could be chosen from the beginning, thus avoiding all the complications. The application of the second consideration generated an acceptable healing rate, but also a considerable disability which affected the restoration of limb function with the use of a prosthetic device.

Clinical parameters are often used in conjunction with non-invasive techniques of circulation to evaluate the extent of arterial blockage. In healthy patients, the peripheral circulation is investigated with the use of the Doppler ultrasonography and the ankle-brachial pressure index. However, in patients with diabetes mellitus, the accuracy of ankle-brachial pressure index is reduced due to the calcification of the walls of the distal arteries, which limits the compressive properties of the vessels, creating an artificially high pressure in the ankle (Goss, 1991). Ballard

et al. (1995) proposed a different modality of evaluating the healing rate of diabetic foot ulcers with potential peripheral ischaemia; namely, to employ photoplethysmography to measure the systolic toe pressure or to determine the distal transcutaneous oxygen tension. These two methods can only be carried out in specialised diabetic foot centres or vascular laboratories and provide a general estimate of the healing rate, prior to the use of angiography.

A considerable number of studies have been conducted to identify the most suitable technique of establishing an adequate amputation level, as well as to weigh the positive and negative aspects of more distal amputations (Pinzur, 1993). Nonetheless, the developments in the field of prosthetics may increase the functionality of more proximal amputations (Pasquina *et al.* 2005).

2.11 Trends in lower limb amputation

Vamos *et al.* (2010) conducted a study on the non-traumatic leg amputations procedures used on patients with diabetes mellitus and patients out with diabetes mellitus in the period 1996-2005, in the UK. The results revealed that a total of about 84,000 patients had undergone 105,193 amputations, of which about 56,000 were minor and 48,569 were major, during 101,115 hospitalizations. Four percent of the patients (4078) had undergone concomitant bilateral procedures. Similarly, López-de-Andrés *et al.* (2011) carried out a study on non-traumatic leg amputations performed on patients with diabetes mellitus and patients without diabetes mellitus during the period 2001-2008, in Spain. It was noted that about 90,000 people had undergone amputations, of which about 46,000 were minor and about 43,000 were major, correlated with about 86,000 discharges. On average about 4% of the patients had undergone concomitant bilateral procedures. Another study done by Ikonen *et al.* (2010) looked at the trends of major amputation in Finland in patients with diabetes mellitus over a decade (1997-

2007). They reported that 9,481 patients had undergone first major amputations during this period. Patients with diabetes mellitus accounted for 53.2% of cases (5,047); of these 973 had type 1 diabetes mellitus and 4,074 had type 2 diabetes mellitus. In their study, Van-Houtum *et al.* (2004) examined the prevalence rate of leg amputations among patients with diabetes mellitus during the period 1991-2000 in the Netherlands and noted that there was a decline in the prevalence rate, from 55.0 to 36.3 per 10,000 patients with diabetes mellitus ($p<0.05$) of both sexes (men – 71.8 to 46.1; women – 45.0 to 28.0).

2.11.1 Trends in lower limb amputation- Patient characteristics

In the study conducted by Vamos *et al.* (2010), 40% of the leg amputations carried out during the period 1996-2005 were performed on patients with diabetes mellitus, of which 13.5% had type 1 diabetes mellitus and 26.5% had type 2 diabetes mellitus. In addition, 66% of patients who had undergone amputation were older than 65 years. Male patients with diabetes mellitus had a higher prevalence rate of limb amputation than female patients with diabetes mellitus, the male-female proportion being two times as large as that of healthy individuals. A considerable decline in the average age at which patients of both sexes had undergone amputation, from 71.2 to 69.4, was also recorded. The percentage of men with diabetes mellitus, as well as without diabetes mellitus, exhibited a growth from 60% to 67%. The prevalence of major limb amputations decreased among patients older than 45 years, but grew among patients younger than 44 years. The estimated average age of patients subjected to leg amputations was 69.5 years; the average age of patients with type 1 diabetes mellitus was 62.09 years, that of patients with type 2 diabetes mellitus was 70.7 years, and the average age of patients without diabetes mellitus was 70.2 years (López-de-Andrés *et al.* 2011). According to Ikonen *et al.* (2010), men were more likely to undergo major amputation at a younger age than women, with average ages of 69.8 and 78.6 years, respectively. Similarly, patients with diabetes

mellitus were more likely to undergo amputation at a younger age than patients without diabetes mellitus, with average ages of 73.2 and 75.5 years, respectively. The results obtained by Van-Houtum *et al.* (2004) were also similar; they observed that the average age at which patients were first subjected to amputation was 71.5 years, which remained constant throughout the period of study. The average age of men subjected to amputation was 69 years, whereas the average age of women was 74.3 years.

Based on the results of the study, Vamos *et al.* (2010) noted that there was a decline in the prevalence rate of major amputations, from 1.3 to 0.7 per 100,000 people in type 1 diabetes mellitus and from 7 to 4.9 per 100,000 people without diabetes mellitus. However, in the case of type 2 patients with diabetes mellitus, the prevalence rate of leg amputations experienced an increase, from 2 to 2.7 per 100,000 people. A similar decline in the prevalence rate of major amputations from 0.59 to 0.22 per 100,000 people in type 1 diabetes mellitus was reported by López-de-Andrés *et al.* (2011) as well. The prevalence rate of major limb amputations in patients with type 2 diabetes mellitus exhibited a more substantial increase, from 7.12 to 7.47. It was estimated that the prevalence rate for first major amputations was 7.4 times higher in patients with diabetes mellitus than the patients without diabetes mellitus (Ikonen *et al.* 2010).

2.11.2 Trends in lower limb amputation- Hospital stay

Patients who underwent major amputations as a result of type 1 and type 2 diabetes mellitus, as well as other diabetes mellitus-related complications, had a longer period of hospitalization than those who underwent amputation as a consequence of other conditions, the average for type 1 diabetes mellitus being 36 days, type 2 diabetes mellitus 37 days and non-diabetes mellitus 30 days respectively (Vamos *et al.* 2010). According to López-de-Andrés *et al.* (2011) length of hospital stay was similar among patients undergoing major lower limb amputation.

According to Van-Houtum *et al.* (2004), there has been a decline in the average length of hospital stay among both sexes during the period 1991 to 2000, from 45 days (44.4) to 36.2 days (SD 38.4).

2.11.3 Trends in lower limb amputation- Mortality rates

In the UK, during the period 2000 to 2004, there was no substantial increase in the mortality rates for the first month and 12 months following the major limb amputation procedures, on the contrary, the rates experienced a decline in this period (Vamos *et al.* 2010). During the period 2001 to 2008, the mortality rate in patients with type 1 diabetes mellitus who had undergone major amputations experienced only a slight increase, from 8.3% to 8.7%; the mortality rate increase was more substantial among patients with type 2 diabetes mellitus, from 9.7% to 10.1%. Among patients without diabetes mellitus who underwent amputation, the mortality rate decreased, from 15.1% to 14% (López-de-Andrés *et al.* 2011). During the first year following the first major amputation, the mortality rate was recorded as being high and varying between 27% and 57%, according to the age of the patients. The mortality rate during the five years following amputation was 60-90% (Ikonen *et al.* 2010).

2.12 Stump healing

2.12.1 Introduction

The time and the ability of a patient to walk with a prosthetic limb who has undergone a lower limb amputation is determined largely by the process of wound healing (White *et al.* 1997). In addition to the type of treatment, the characteristics of the wound and the condition of the patient also influence the stump healing process. Pino *et al.* (2011) concluded, after reviewing 19 studies about lower limb amputation in patients with diabetes mellitus, that complete preoperative workup is desirable before an amputation and emphasis should be put on

evaluation of probable rate of healing, the functioning condition of the limb prior to surgery, control or treatment of any additional diseases that the patient might suffer from, as well as selection of the level of amputation based on up-to-date techniques.

2.13 Complications of stump healing

Lower limb amputation surgery is a major surgery done in patients who have multiple co-morbidities. A large number of patients with diabetes mellitus are admitted to hospital due to lower limb-related problems (Boulton *et al.* 2005). McIntosh *et al.* (2009) carried out a retrospective study from 2005 to 2007 in 231 patients who underwent major amputations of lower extremities. They observed that 7.3% of the patients experienced wound infection, phantom pain, poor body image, depression and myocardial infarction following major amputation procedures.

2.13.1 General complications

Diabetics are two times more likely to experience congestive cardiac failure after amputation than patients without diabetes mellitus and the risk for deep vein thrombosis (DVT) is 11% (Schofield *et al.* 2006).

2.13.2 Local complications

2.13.2.1 Infection of the stump

Patients who have undergone amputation can develop severe problems as a result of infection, especially if they suffer from diabetes mellitus. The amputated stump frequently becomes infected requiring re-amputation (Godoy *et al.* 2010). The wound infection rates following major lower limb amputation have been indicated to vary between 13% and 40%. *Methicillin-resistant Staphylococcus aureus* (MRSA) was the most common infection causing organism

(Godoy *et al.* 2009). The morbidity and mortality rates usually increase as a result of MRSA infection in vascular patients (Malde *et al.* 2006).

As indicated by Ray (2000), patients with diabetes mellitus are five times more predisposed to wound infection than patients without diabetes mellitus. The presence of peripheral vascular disease increases the risk of infection even further. In a study by Aulivola *et al.* (2004), infection developed in 5.5% of cases of trans-tibial amputations and 6.7% of cases of trans-femoral amputations. A wound infection can produce excess discharge and disrupt the suture line (Stringfellow *et al.* 2000). Baxter (2003) indicated that an extensive infection can even generate wound rupture and tissue death, requiring additional surgical interventions. According to the Infection Surveillance Service in England (Infection Surveillance Service, England 2006), leg amputations are associated with the highest risk of infection, because many patients are subjected to this procedure as a consequence of severely infected ulcerations. Additionally, there are several factors which may increase the likelihood of infection, such as inadequate blood circulation, weak immune system and poorly controlled diabetes mellitus.

2.13.2.2 Pain in the stump

Pain is a significant problem in lower limb amputation wounds and can be very complex to deal with. Incision stump pain and phantom limb pain are the two kinds of pain that patients who have undergone amputation suffer from. Stump pain occurs only in the area closely surrounding the stump and amputation site (Ellis, 2002). If left untreated, stump pain can adversely influence the wound healing process and, consequently, reduces the quality of life of the patient. It has been demonstrated that opiates and non-steroidal anti-inflammatory agents can alleviate stump pain.

2.13.2.3 Tissue necrosis of the stump

As a large number of amputations are performed due to ischaemia, inadequate circulation in the stump area can cause tissue necrosis in the aftermath of the amputation procedure. Dead tissue manifests as changes in skin colour, dry gangrene, or wet gangrene. Ray (2000) pointed out that changes in skin colour around the incision line can determine wound rupture following surgical intervention or tissue death in areas that are not viable a number of weeks after the procedure.

Debridement is a good technique to accelerate wound healing (Harker, 2006). Dead tissue present in reduced amounts is left to be removed through the process of autolysis, once it is ascertained that it does not pose any danger. But in cases of significant necrosis, wound debridement is preferred. A decision must be made as to which method is best suited for the removal of dead tissue (Flanagan, 1997). A number of factors have to be taken into consideration at the time of making this decision, including convenience, wound type, location, and expenses (NICE Guidelines, 2015). The use of sterile maggots, known as larval therapy, is one method of debridement that is frequently employed in the United Kingdom. The preponderant use of this method is attributed to the fact that it is the only viable option in most cases, the presence of co-morbidities preventing surgical revision or the use of other methods of stump debridement (Jones *et al.* 1999).

2.13.2.4 Stump oedema

Stump oedema is a common problem faced after lower limb amputation especially in patients who are suitable for prosthesis fitting. According to Ray (2000), among the factors believed to cause extensive and protracted oedema are pre-existing venous deficiency, generalised fluid retention - normally as a result of congestive heart failure, and chronic hypervascularity. The

latter is frequently encountered in patients with diabetes mellitus without a severely disrupted circulation.

2.13.2.5 Osteomyelitis

Osteomyelitis remains a dreaded complication post amputation as it can result in life threatening sepsis (Kapoor *et al.* 2007). The bone in the amputation area can become exposed as a consequence of muscle withdrawal over the stump and exposure of the underlying bone through the skin (Ray, 2000). A ruptured wound can facilitate bone exposure, as well, increasing the risk of osteomyelitis. If the extent of exposed bone is considerable and the granulation tissue cannot cover it through secondary healing, surgical intervention is needed.

2.13.2.6 Haematoma in the stump

Bale *et al.* (1997) defined a haematoma as a localised accumulation of blood inside an organ, cavity or tissue. A haematoma provides a suitable environment for the development of infection and can generate dead space, undermining the suture line and expanding the level of tension in the wound (Baxter, 2003). Haematomas usually drain freely and do not necessitate surgery. Nevertheless, surgical debridement is employed to remove considerable quantities of coagulated blood (Ray, 2000). Morrison *et al.* (1997) highlighted the fact that there is an increased likelihood of haematoma formation under the suture line in the case of wounds without drainage, which can lead to the development of tension, oedema and infection. Furthermore, the blood circulation can also be affected by the increased tension under the suture line, causing wound rupture and tissue death (Partridge, 1998). In order to identify a haematoma, efficient evaluation methods should be employed, and the patient must be referred if warranted to surgery immediately upon discovery of haematoma.

2.13.2.7 Wound dehiscence

Wound dehiscence usually happens suddenly along the suture line and is accompanied by a sharp rise in sero-sanguineous drainage (Heller *et al.* 2006). It comes to pass in cases where the wound is too weak to resist any exterior forces applied to it such as shear or direct trauma (Bale *et al.* 1997). Wound dehiscence also occurs as a result of premature removal of sutures or stump oedema which creates tension in the wound. Total dehiscence can potentially determine exposure of muscle and bone (Baxter, 2003).

2.13.2.8 Non-healing requiring a higher level amputation

Re-amputation refers to a revision of an amputation to a higher level secondary to non-healing of the stump. There are a number of factors which may substantiate the need for re-amputation, such as stump pain and/or phantom limb pain, delayed stump infection, the formation of symptomatic bone spurs, assessment of the skin flap designed to preserve stump length, and preparation of the stump for the prosthetic device. Dillingham *et al.* (2005) observed that re-amputation is more likely to occur in patients with diabetes mellitus than in patients without diabetes mellitus. Reiber (2001) estimated that 9% to 20% of patients with diabetes mellitus with an initial leg amputation undergo re-amputation within the first year, and 28% to 51% of patients with diabetes mellitus necessitate re-amputation within five years of the original amputation.

2.13.2.9 Death following inability to heal stump

Criqui *et al.* (1992) revealed that the likelihood of patients with peripheral vascular disease who had undergone lower limb amputation to die because of cardiovascular complications within ten years of the amputation is six times higher than that of patients without peripheral vascular disease. Lee *et al.* (1993) reported that the most common cause of death after a lower

limb amputation was diabetes mellitus (37.3%), cardiovascular disease (29.1%), and renal disease (7.3%). According to Mayfield *et al.* (2001), the mortality rate among patients with renal disease, cardiovascular disease or proximal amputation level was high during the first 12 months of the procedure. Toursarkissian *et al.* (2002) indicated that in the period immediately following the amputation, after one year and after five years of the procedure, the mortality rates were up to 23%, 41% and 80%, respectively.

The five-year mortality rates associated with above-knee amputation and below-knee amputation were estimated at 90% and 70%, respectively (Hambleton *et al.* 2009). Tentolouris *et al.* (2004) found that a similar percentage (61%) of patients with diabetes mellitus with ages between 67 and 76 years who had undergone amputation, were likely to die within 5 years of the operation. Heikkinen *et al.* (2007) reported that, despite being younger, the mortality rate among patients with diabetes mellitus with amputations was much higher than the mortality rate of patients without diabetes mellitus post amputations. According to Schofield *et al.* (2006), the mortality rate among patients with diabetes mellitus with amputations was 55% higher than among patients without diabetes mellitus. One reason for the high mortality rate may be the emphasis put on rescuing the limb and amputation being resorted to only when revascularization is not feasible.

2.14 Prediction of stump healing

2.14.1 Introduction

The crucial part of any lower limb amputation is the stump healing process, which represents the central goal of post-surgery management. Despite the importance attributed to this process, a standard set of guidelines regarding efficient healing methods is yet to be formulated. The prognosis of the stump healing rate is fraught with considerable difficulties since this process

is influenced by a number of factors in addition to blood circulation, including nutrition, surgical methods, post-surgery treatment, development of infection, and stump trauma. According to Chalmers and Tambyraja (2002), no prediction of stump healing is completely accurate. Nawijn *et al.* (2005) added that a general rule for the prediction of stump healing is yet to be formulated.

2.14.2 Blood markers and factors

Several factors can influence the healing of a stump after a lower limb amputation as discussed below.

2.14.2.1 Diabetes and glycaemic control (HbA1c) as a marker for lower limb amputation

As pointed out by Imran *et al.* (2006), poor diabetes mellitus control increases the likelihood of amputation among patients with diabetic foot disease, and underpins vascular and neuropathic complications. The association between amputation and elevated glycaemic levels is well documented (Brownlee, 2005). Among the causes of vascular insufficiency, diabetes mellitus remains the main contributor to the rates of lower limb amputation. HbA1c which estimates the average glucose reading in the last 3 months is a useful and effective indicator of diabetes mellitus control. Several studies have shown that improving glycaemic control could help healing of the diabetic foot ulcer.

2.14.2.2 Age as a patient marker for lower limb amputation

According to Gilliver *et al.* (2007), it is possible that the healing of acute wounds is adversely affected by internal aging processes. Ashcroft *et al.* (1998) have highlighted the impact on age-related debilitated healing process exhibited by prolonged inflammation, up-regulated protease action, and decreased matrix generation.

Older patients are more likely to experience limited wound healing as a result of physiological changes associated with aging, such as reduced skin elasticity and loss of collagen (Van De Kerkhof *et al.* 1994). In addition, there is a higher risk of infection among older individuals as their immunity is reduced. The circulation and oxygenation of the wound can be negatively influenced by the presence of other chronic diseases. In their study on older patients suffering from diabetes mellitus, Liu *et al.* (2008) observed that the wound healing process was also slowed down by the decrease in the production of transcription factor Hypoxia-Inducible Factor 1α.

2.14.2.3 Gender as a patient marker for lower limb amputation

Wound healing in older patients is also subject to the influence of sex steroid hormones, which have been shown to determine an inflammatory reaction *in vivo*. This can be kept in control with the use of topical and systemic oestrogen therapy, which has also enhanced the rate of acute wound healing in both sexes, but more pronounced in older women (Ashcroft *et al.* 2002). Dehydroepiandrosterone has been indicated to have a similar effect, due to the fact that it can be converted to oestrogen (Mills *et al.* 2005). On the other hand, Ashcroft *et al.* (2002) demonstrated that the male produced testosterone hormone slows down endogenous wound healing in the elderly. They explained that this is by a direct upregulation of proinflammatory cytokine expression by macrophages in response to testosterone. This may be the reason why old men exhibit a lower rate of wound healing than old women, as the level of testosterone in men may remain quite high despite aging. However, other studies including the one undertaken by Demling and Dennis (2000) argued that anabolic agents like oxandrolone which is a testosterone analogue improved wound healing in patients with severe burns compared to a placebo group (13 ± 3 days to 9 ± 2 days) in a randomized double-blinded placebo-controlled study. Labrie *et al.* (2001) argued that reduced wound healing in older patients is caused by the

loss of the protective action of oestrogen and Dehydroepiandrosterone (DHEA), as their levels diminish with age. According to Ashcroft *et al.* (2003), who did extensive research on the role of sex hormones on wound healing, the effect of oestrogen on cutaneous wound healing is expressed as adjustment of the inflammatory reaction, cytokine activity and matrix generation, as well as enhancement of the process of re-epithelialization, angiogenesis and the control of proteolysis. Oestrogen attenuates localised inflammation by limiting invasion of inflammatory cells and hindering the production of pro-inflammatory cytokines. An increased production of elastase was found to stimulate extensive tissue destruction associated with chronic wounds; oestrogen restricts neutrophil migration and the production of neutrophil-derived elastase, thus reducing extensive tissue destruction (Herrick *et al.* 1997). Taylor *et al.* (2002) reported that older patients are affected in a proportion of 70% by leg ulcers. Decrease in the levels of oestrogen and Dehydroepiandrosterone that accompanies aging may partly account for this high incidence of chronic wounds in the elderly.

2.14.2.4 Kidney function (blood urea, serum creatinine and serum electrolytes) as a marker for lower limb amputation

Acute renal failure results when there is an insult to the kidneys thereby increasing the urea and serum creatinine and resulting in a decline in the glomerular filtration rate. It has an impact on wound healing in various ways. According to Druml (2005) renal failure is associated with fundamental alterations of metabolism and immunocompetence including the induction of a pro-oxidative and pro-inflammatory state which results in a poor healing process. Janssen *et al.* (2002) and Okada *et al.* (2003) conducted molecular analyses on patients with diabetes mellitus suffering from renal disease and discovered that inflammation is an integral part of diabetic nephropathy, alongside activation of protein kinase C, the production of advanced glycation end products and excessive manifestation of the Transforming Growth Factor (TGF)-

β (Brownlee, 2001). During the period 1996-2005, Akha *et al.* (2010) examined 244 patients with kidney disease and diabetic foot ulcers, observing that the patients who had undergone leg amputation had a high serum creatinine. Patients with End Stage Renal Disease secondary to diabetes mellitus have a high incidence rate of peripheral neuropathy and peripheral vascular disease, both being significant risk factors for leg amputation and frequently accompanied by uraemia (Fernando, 1991). The results of the three year study conducted by Eggers *et al.* (1999) on End-Stage Renal Disease patients subjected to amputation revealed that End Stage Renal Disease had an adverse effect on the wound healing process and was associated with high rates of morbidity and mortality following the amputation procedure.

2.14.2.5 Infection markers (white cell count, C-reactive protein) as a marker for lower limb amputation

One of the common causes of delayed wound healing is infection of the wound. There are a number of factors involved in the development of wound infection, including the number and type of bacterial growth, the reaction of the host's immune system, as well as the virulence and synergistic action of the different bacterial species. This infection could be at many levels. If the infection spread to the ligaments, tendons and bones, septic thrombosis and gangrene can develop independent of macroangiopathy. According to de Godoy *et al.* (2010), who reviewed 231 patients retrospectively from 2005 to 2007, infection of stump wound had a high incidence rate of up to 40% and caused impaired healing, thus requiring a re-amputation. It has been argued that there is a delicate balance between the mechanisms of protection activated by phagocyte invasion and the conditions which stimulate exaggerated invasion, which, if disrupted, impedes wound healing. Dovi *et al.* (2003) noted that patients with peripheral vascular disease and diabetes mellitus often experience chronic wound recurrence as a result of excessive leukocyte invasion which determines extensive production of proteolytic

enzymes, oxygen-free radicals and pro-inflammatory cytokines. The wound healing process is also slowed down by disruption of leukocyte recruitment (Miller *et al.* 2006). According to Kim *et al.* (2008) protein-polymorphonuclear leukocyte (EGFP-PMN) and neutrophils' entry into the healing site play a key role in wound closure, which can be compromised in patients with diabetes mellitus secondary to intracellular hyperglycaemia. According to Nather *et al.* (2008), the type of organism causing the infection is also important as some pathogens, such as *Methicillin-resistant Staphylococcus aureus*, represented a risk factor for amputation in patients older than 60 years.

Apart from being a marker of inflammation, C-reactive protein plays an essential role in the innate immune system of the host and contributes to protection against autoimmunity. It is one of the most important proteins that is rapidly produced by hepatocytes during an acute-phase response upon stimulation by Interlukin- 6 (IL-6), Tissue Necrotic Factor- α (TNF-α), and Interlukin-1- β (IL-1-β) originating at the site of inflammation or pathology (Vermeire *et al.* 2004)

2.14.2.6 Coagulation profile (International Normalised Ratio/Prothrombin Time) as a marker for lower limb amputation

Immediately after a wound is formed, platelet aggregation and haemostasis are activated to prevent local haemorrhage, and white blood cells, fibroblasts and blood vessels migrate to the wound area to begin the healing process (Laurens *et al.* 2006). The activity of the blood platelets is stimulated by endothelial injury and they form a platelet plug, known as primary haemostasis process, which halts bleeding. At the same time, the process of coagulation is activated, transforming soluble fibrinogen to a system of insoluble fibrin fibres and providing stability to the platelet plug by creating a more extensive system which incorporates platelets

through the adherence of fibrin to the activated platelets receptors (Fang *et al.* 2005). The latter produce a series of growth factors such as the platelet-derived growth factor. In turn, the growth factors prompt the fibroblasts to generate collagen, glycosaminoglycans and proteoglycans, thus advancing the process of healing (Brissett *et al.* 2003). The fibrin matrix has a role in arresting the haemorrhage, as well as in tissue regeneration, leukocyte cell attachment, and the movement of endothelial cells during the process of angiogenesis. After a series of cascading events, a fibrin clot is formed, and it is soon invaded by the phagocytes. Chemo-attractant factors, such as fibrinopeptides, divided from fibrinogen by thrombin, as well as collagen and enzymatically active thrombin, control the infiltration of granulocytes and monocytes into the wound area. Laurens *et al* (2006) concluded that fibrinogen and thrombin play an important role in wound healing.

2.14.2.7 Smoking as a marker for lower limb amputation

Many researchers (Harvey *et al.* 2002; Hoogendoorn *et al.* 2002) have argued that smoking slows down healing and increases the risk of infection and osteomyelitis. Ueng *et al.* (1999) observed that vascularisation in areas of bone healing is considerably diminished by nicotine, thus slowing down healing. According to Castillo *et al.* (2005) who carried out a retrospective multivariate analysis on the impact of smoking on wound healing in lower limb fractures concluded that non-smokers were more likely to heal a wound site in comparison to smokers (40.1% vs 42.9%). They also added that non-smokers were also less likely to develop infections (14.8% vs 24.8%) and osteomyelitis (4.9% vs 17.1%). According to Vanross *et al.* (2009) smoking and delayed mobilisation after lower limb amputation impeded stump healing. The detrimental effect of smoking on wound healing is due to the toxic components of cigarettes, especially nicotine, carbon monoxide, and hydrogen cyanide. Nicotine, as a vasoconstrictor, can contribute to the development of ischaemia and poor wound healing as it decreases

cutaneous circulation. It can also increase the likelihood of formation of thrombotic microvascular occlusion as it amplifies the adhesive properties of platelets. Silverstein (1992) noted that nicotine also decreases the production of erythrocytes, fibroblasts and macrophages. Carbon monoxide which is noted to be in higher quantities in smokers, has an adverse effect on oxygen transport and metabolic activity, and also prevents the action of enzymes, which is required for the oxidative metabolic activities and oxygen transport to the cells.

2.14.2.8 Cholesterol markers (serum cholesterol, serum Low Density Lipids, serum High Density Lipids) as a marker for lower limb amputation

Physiological and pathological processes, such as angiogenesis, reconstruction of arterial lesions and vascular graft healing, depend on endothelial cell migration. Oxidised Low-Density Lipoproteins (LDL) stimulate the intracellular development of reactive oxygen factors in endothelial cells, which in turn prevents their migration resulting in impaired wound healing (van Aalst *et al.* 2004). According to Rosenbaum *et al.* (2012), hypercholesterolaemia is associated with increased oxidative stress, which disrupts the activity of endothelial and smooth muscle cells, impairing the healing process following arterial injury. Cakmak *et al.* (2009), based on a histopathological analysis, argued that the administration of Simvastatin, which reduces the level of cholesterol, enhances the healing process by stimulating re-epithelialization, limiting the development of granuloma and ischaemic necrosis, and reducing the spread of inflammation to the muscles. It was noted that abnormal lipid profile not just plays a negative role in atherosclerosis but also delays wound healing.

There are other methods employed to estimate stump healing. As pointed out by Davis *et al.* (2004), most of the methods that have been proposed for estimating the healing rate of stump wounds entail the evaluation of the distal circulation. Toe pressures have been demonstrated to

correspond to the healing potential of a leg amputation (Ballard *et al.* 1995). Malone *et al.* (1987) have argued that the ankle brachial pressure index, intra-dermal xenon-133, or absolute popliteal artery Doppler systolic pressure values do not generate an accurate prognosis of stump wound healing. What is more, Doppler pressures and ratios were demonstrated to be incapable of distinguishing between unsuccessfully and successfully healed trans-tibial amputations (Wagner *et al.* 1988). This may be due to the development of arterial media calcification present in diabetic vessels, which has a detrimental effect on vessel compliance and the possibility of occluding the artery with the use of an external cuff, thus determining an excessively high arterial pressure. Pino *et al.* (2011) recommended increasing transcutaneous oxygen pressure while breathing 100% oxygen in a hyperbaric chamber to generate a prognosis of healing rate. Similarly, Vanross *et al.* (2009) recommended the increasing transcutaneous oxygen tension (TcPo2), which is a non-invasive method that can provide an effective indication of the likelihood of stump healing for the fitting of prosthetic limb in below-knee amputations.

Another method of estimating stump healing is the tactile evaluation of skin temperature. Henderson *et al.* (1978) observed that the use of thermography could enhance the accuracy of determining amputation level in one third of the cases studied. Thermography was employed by Spence *et al.* (1981) as well, who obtained an 80% success rate, which was similar to that attained by clinical impression alone. Among all the non-invasive methods that were assessed by Wagner *et al.* (1988), it was proposed that at a temperature of 90°F (32.2°C), the skin exhibited the most efficient combination of sensitivity, specificity, accuracy and prognosis. These results could be further enhanced by comparing the temperature of the skin to that of the surrounding environment.

As noted by Martinez-Hernandez (1988), an essential component of the wound healing process is vascularisation. The suitability of perfusion has been indicated as particularly essential for

the healing process (Harker, 2006). The circulation in the wound area is a significant indicator of healing. There is no method of evaluating peripheral circulation that has received unanimous approval and clinical judgement alone is not enough to generate a prognosis of the healing rate of an amputation (Sarin *et al.* 1991). Angiography was indicated by Solakovic *et al.* (2008) as being suitable for estimating the amputation level. This affirmation was opposed by Huber *et al.* (2003), who argued that angiography is basically a morphological method and despite providing abundant anatomical information, it cannot demonstrate functional circulation.

However, the success of an amputation procedure depends largely on the proper selection of the amputation level, in accordance with evaluation of limb perfusion and limb functionality. Healing and fitting of prosthetic limb also depend on nutrition, age of the patient, the existence of co-morbidities, particularly renal failure, diabetes mellitus and anaemia, as well as on the surgical procedures employed (Taylor *et al.* 2005). Regarding below-knee amputation, there is no particular surgical method that has an influence on wound healing, development of infection, likelihood of re-amputation or functionality of prosthetic limb (Tisi *et al.* 2004). However, Chen *et al.* (2008) argued that the use of a suitable surgical procedure, as well as post-operative wound care, can enhance wound healing and overcome the effect of the unalterable factors such as amputation level, age, and the presence of co-morbidities, including End Stage Renal Disease, dementia, and coronary artery disease. The factors identified by Eneroth (1999) as having a negative impact on stump healing were smoking, reduced haemoglobin and/or haematocrit, inadequate nutrition, diabetes mellitus and no administration of prophylactic antibiotics. It has been estimated that a patient has to have at least 1500 cells/mm lymphocyte count to ensure the successful completion of the stump healing process (Ballard *et al.* 1995). Naidu *et al.* (2005) suggested that the prognosis of stump healing in patients with below-knee amputation can be made based on the existence of microvascular

changes, such as thickening of the inner walls and thinning of the medial walls of the arteries.

Biomarkers provide a dynamic and powerful approach to understanding the spectrum of any disease with applications in observational and analytic epidemiology, randomized clinical trials, screening and diagnosis and prognosis. A good biomarker is relevant to the study, cost effective, easily reproducible, has a high sensitivity and specificity, validity proven towards that disease and least prone to measurement errors and bias. The markers used in this study are factors that play a role in healing of a diabetic foot ulcer as noted in several studies and play a role in the pathophysiological pathways of peripheral vascular disease and diabetes which are the main causes for a lower limb amputation surgery. These biomarkers are easy to use, readily available for a surgeon/physician on a day to day basis for decision making, are cost effective and do not require any equipment or any expertise to use or interpret. The blood markers used are readily available via the laboratory in any hospital setting almost on a daily basis for any inpatient who has regular blood check for his illness.

CHAPTER 3

METHODOLOGY

3. Methodologies

3.1 Research design

This study was a single centre exploratory study. The aim of the study was to evaluate risk factors and blood markers in predicting the results of stump healing following a major (above knee and below knee) lower limb amputation surgery. This study was divided into two parts, namely retrospective and prospective. In the first part, to develop a predictive model, data was obtained from patients who underwent a major (above knee and below knee) amputation at the Royal Infirmary of Edinburgh. The data collated included 300 lower extremity amputees, who underwent amputation (above or below knee) surgery between 2006 and 2009. One hundred patients who underwent major (above knee and below knee) amputation surgery between 2010 and 2011 were included in the prospective part of the study. This latter data was used to validate the model that had been developed from the retrospective data. This method of development and validation of prediction model was adopted to estimate the probability of developing a particular outcome in the future (which was stump healing in this study) (Collins *et al.* 2011). Predictive tools, such as this model, are not a substitute for clinical assessment but are intended to be supplementary and reinforce medical opinion. As reported by Ross *et al.* (2002), prediction models deliver enhanced reliability and accuracy compared to forecasts based on subjective evaluations.

External validation of the predictive performance of a prediction model which is an evaluation of a predictive model, in datasets that were not used to develop the model, is critical prior to employing it in the clinical setting (Steyerberg *et al.* 2009). Externally validating the model reflects its effectiveness and ability to differentiate between the development and validation cohorts. The model's performance evaluation involves assessing calibration and

discrimination. Therefore, the model must be empirically appraised using data separate to that which was used to create the model (Steyerberg *et al.* 2013). This empirical appraisal highlights any shortcomings in the statistical models used in developing this predictive model as well as gauging the potential for using this model in different clinical settings.

3.2 Sample size of the retrospective and prospective study

The purpose of this exploratory research study was to explore associations between lower limb stump healing defined as complete closure of the wound adequate to take a prosthesis for functional/cosmetic purposes (dependent variable) and biomedical variables (independent variables) in predicting stump healing.

Logistic regression predictive models can be used to help determine the prognosis for stump healing (healed/not healed). For a statistical study to be successful and achieve its objectives, analysis of the sample size is critical. For binary logistic regression, effective sample size can be estimated by the number of events or non-events (van Houwelingen and le Cessie, 1990). In other words, the number of healed/not-healed events can be used to guide the effective sample size suitable for logistic regression.

Vergouwe *et al.* (2005) recommended that, ideally, at least 100 events and 100 non-events should be used to ensure that the external validation studies are accurate. Therefore, a model should be developed from a base of no fewer than 100 events and 100 non-events to ensure adequacy of power. This recommendation has been based on the observation that samples consisting of approximately 100 events were able to identify considerable discrepancies in model efficiency in almost 80% of cases. The proportion of events (stump healing rates) varied greatly in previous studies and the lowest healing rate was taken in order to have enough patients to power the study which was 30% (Dormandy *et al.* 1999). This would mean that a

sample size of 100 patients would provide 30 events and in order to obtain 100 events, a sample size of 330 would be needed to adequately power the model. According to Vergouwe *et al.* (2005) statistically significant variations in model performance can be highlighted from an approximation of 80% power provided by an initial sample of 100 events. As a generalisation, prognostic models tend to be overly optimistic and as such, do not always work well in practice.

Both the condition of the patient and the model configuration dictate the development and the external validity of a model. Prognostic models do not always work well in practice, so it is widely recommended that they need to be validated in a new patient dataset (Altman and Royston, 2000). The development process of the model and the subsequent new patient set (for the prospective study) to which the model is applied contribute to the model's external validation. In this instance, data was collected from 400 patients; 300 patients were recruited to develop the model (retrospective study) and 100 for the model validation (prospective study) (Stone, 1974).

3.3 Ethical approval of the retrospective and prospective study

This study was reviewed and given favorable ethical opinion by the South East Scotland NHS Research Ethics Committee, NHS Research and Development and the Queen Margaret University Research Ethics Committee (Appendix I). Approval of the Ethics Committee was obtained for both the Retrospective and Prospective part of the study.

3.4 Consent process

3.4.1 Consent process for the retrospective study

Consent for the retrospective study was not required as the data collected was anonymised and the data collection link was broken.

3.4.2 Consent process for the prospective study

The welfare and security of patients and their information were paramount. In accordance with ethical guidelines, participating patients were recruited as volunteers, fully aware that they were free to withdraw from the study at any time without reason and without penalizing their treatment. To enable patients to make an informed decision about being a participant in the study, detailed information was provided to each one (Appendix III) and enough time was given for the participants who were interested in the study to reading and understanding the Patient information sheet with a subsequent session booked if the participant had any questions about the study before giving consent. Patients gave written informed consent to become a participant and where this could not be obtained, the patient was excluded from the study (Appendix IV).

3.5 Retrospective study (development of prognostic model)

The potential participants were retrospectively purposively chosen by the researcher on the basis of the inclusion and exclusion criteria from those who underwent a below or an above knee amputation procedure at the Royal Infirmary of Edinburgh.

3.5.1 Inclusion and exclusion criteria

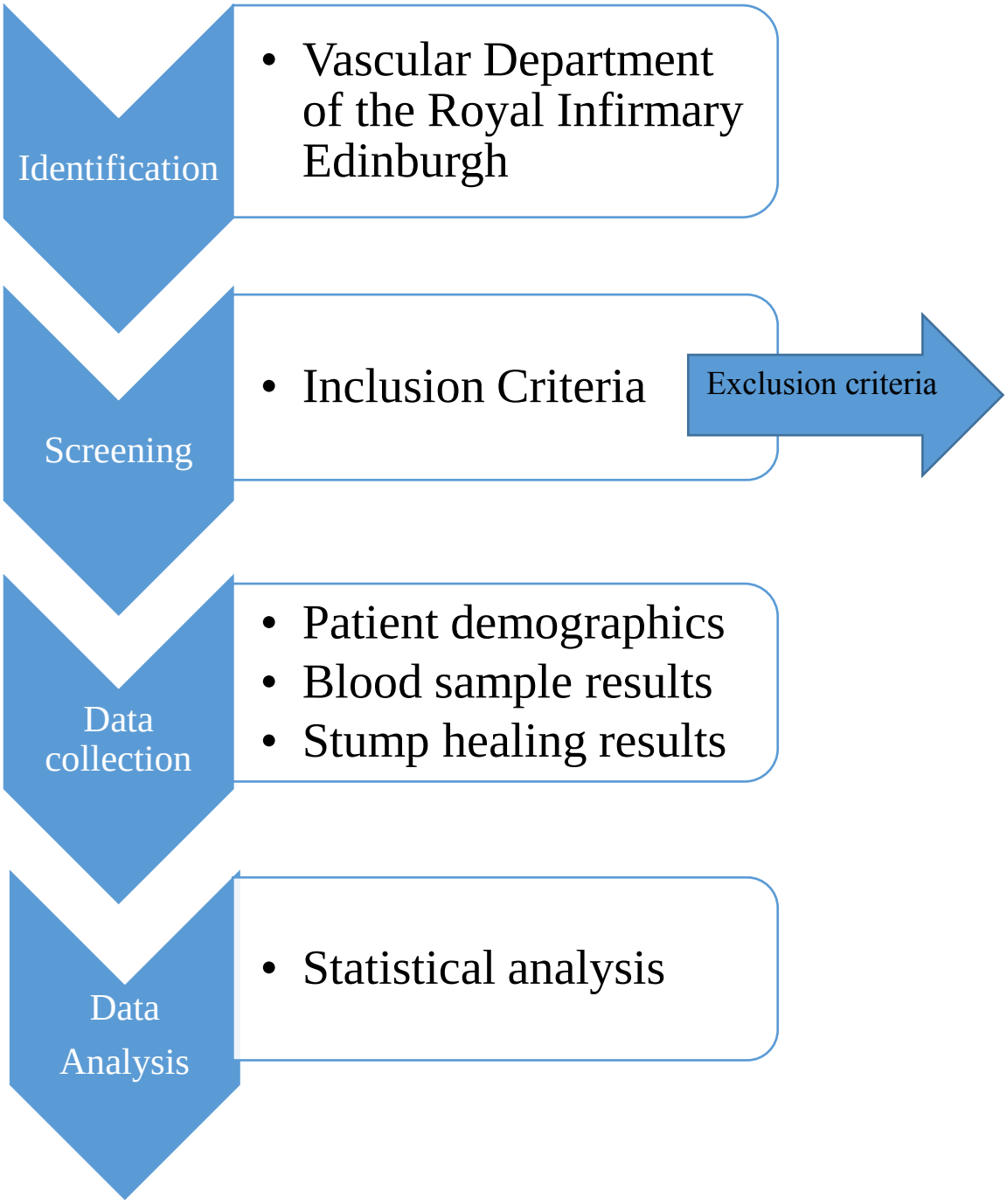
3.5.1.1 Inclusion criteria:

- Patients whose age was 18 years and above.
- Both genders (male and female) were eligible for the study.
- Patients who had an amputation at a level of below or above knee for the first time.

3.5.1.2 Exclusion criteria:

- Patients who had a revision of their stump either at the same level or at a higher level.
- Patients who had a traumatic amputation due to an accident.
- Patients who had did not have complete information available required for the study.

Figure 8: Flow diagram showing steps of retrospective study from recruitments of subjects to data analysis.



A list detailing patients who had undergone above or below knee amputation surgery was initially obtained from the Department of Vascular surgery, Royal Infirmary of Edinburgh. To recruit patients a selective sampling technique was used. The hospital's vascular database was probed for a retrospective case note review of patients who had received amputation surgery.

Case files were obtained from the medical records (Proton® software, and Apex® software) for those patients whose amputation surgery was conducted at the Royal Infirmary Edinburgh between 2006 and 2009. Analysis of potential participants was undertaken through interrogation of the data. Patients' pre-operative assessments, the clinical outcomes of their operation and follow up case notes were all utilised to compile a full report on each patient.

A total of 384 lower limb amputation surgical procedures were performed during the period. From these 300 cases were selected who underwent either a below knee or an above knee amputation surgery for the first time. Patients were excluded due to incomplete information in relation to the stump healing outcomes (n=36) or those who had a revision of their stump either at the same level (n=31) (BKA followed by a revision at the same level) or at a higher level (n=17) (BKA followed by an AKA).

To perform the retrospective study, a systematic process was employed; this entailed identifying, screening and recruiting participants followed by collecting their data for statistical analysis (Figure 8).

3.6 Predictive measure - stump healing

Stump healing was defined as the complete painless closure of the wound/stump fit enough to take a prosthesis for functional purposes (Tisi *et al.* 2008). It was determined at the end of 12 weeks from the day of lower limb amputation surgery by the vascular consultant in the Royal Infirmary of Edinburgh (Vigier *et al.* 1999; Wong *et al.* 2000; Nawijn *et al.* 2005).

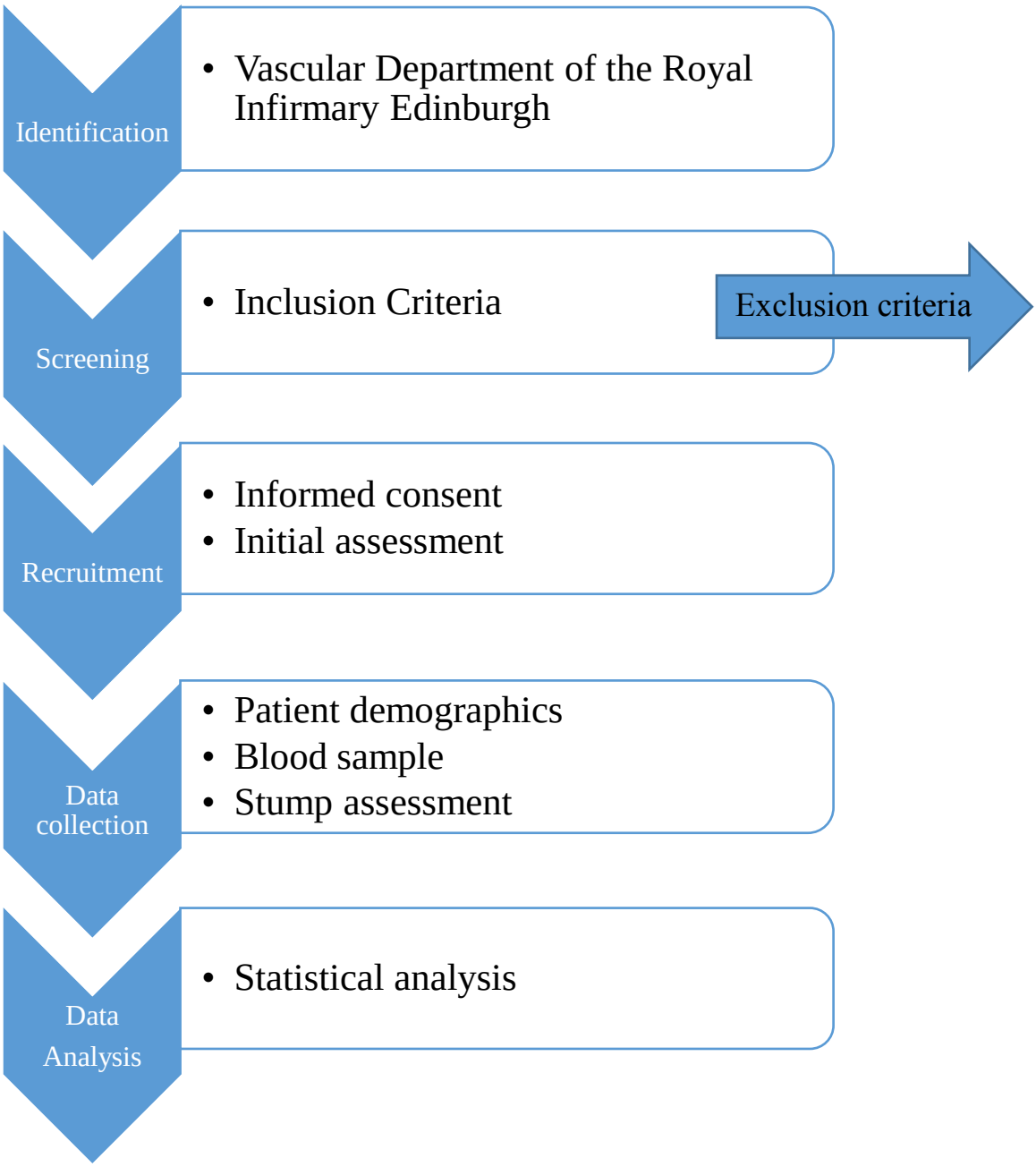
3.7 Data collection

A comprehensive review of the patient's inpatient and outpatient records including the medical and surgical history was performed. The relevant demographic and clinical data collected from

all medical and surgical consultations was examined and entered into an Excel spreadsheet (Microsoft, USA). The Principal investigator was aware about the identity of the patients in order to access the patients' notes. All data was collected as per the Caldicott principles.

3.8 Prospective study (validation of prognostic model)

Figure 9. Flow diagram showing steps of prospective study from identification of subjects to data analysis.



The prospective study employed the same systematic process as used for the retrospective study, i.e. the identification, screening and recruitment of participants was followed by subjecting the collected data to statistical analysis using suitable statistical tests. (Figure 9).

3.8.1 Inclusion and Exclusion criteria

3.8.1.1 Inclusion criteria

- Aged 18 years and older.
- Both genders (male/female) were eligible for the study.
- A planned procedure for first major (below or above knee) lower limb amputation.
- Patients who could manage a follow up period for at least three months.
- Ability to give informed consent.

3.8.1.2 Exclusion criteria

- Patient who had a revision of their stump either at the same level or at a higher level.
- Participant's refusal.
- Patients who had a traumatic amputation due to an accident.

3.8.2 Recruitment of participants

The secondary objective of the study was to validate the regression model by recently treated patients who had a lower limb amputation from 2010 to 2011. The potential participants were selected by the consultant vascular surgeon based on the inclusion and exclusion criteria from the vascular clinic and the vascular ward in the Department of Vascular Surgery at the Royal

Infirmary of Edinburgh.

The Consultant then introduced the Principal investigator who was the PhD student who was conducting the study to the potential participants who were then invited by the Principal Investigator to take part in the research when they attended the vascular clinic or when they were admitted into the vascular ward. Patients who showed an interest were given an information pack which contained patient information sheet and the consent form. They were given at least 24 hours to read the information pack. Those interested in participating then contacted the principal investigator. The principal investigator then went through the patient information sheet and the consent form in detail with the participant. The patient information sheet included the contact details of the researcher and an independent advisor if the potential participants wished to know further details about the study (See Appendix III). Patients who had a revision of their stump or a higher level amputation within 12 weeks of the first surgery were excluded from the study.

According to Stone, (1974) in regression analysis the sample size for model validation should be ideally one third of the sample size for model development. The purpose of the recruitment process for the prospective study was to recruit 100 patients. A total of 145 patients were approached to take part in this study by the principle investigator for prospective clinical research who had undergone below or above lower limb amputation surgery at the Royal Infirmary of Edinburgh between the years 2010 and 2011 in order to recruit 100 patients. Of these, 17 did not want to participate and 18 were excluded as they did not meet the inclusion criteria; 10 participants subsequently dropped out at follow-up. Personal commitments were given as the prime reason for not wanting to take part. Exclusion criteria included the inability to give informed consent (n=4) (non-native English speakers) and second major amputation of

the same leg (n=14) (revision at the same level of the initial amputation or at a higher level). A total of 100 patients were recruited into the prospective study.

3.8.3 Data collection

After taking informed consent, patient assessments were conducted at least 24 hours prior to their surgery. For this pragmatic study, and to ensure the validity of the data, the researcher was present throughout the process and all procedures were undertaken by NHS-accredited staff. The researcher additionally ensured that standard NHS operating procedures were utilised and that the collection and use of patient data was fully compliant with the relevant NHS regulations. The following paragraphs describe the process.

Before, during and after each assessment, every participant was given the opportunity to ask questions and raise their issues. The following baseline demographic information was collected: age, gender, amputation history, risk factors including, diabetes mellitus, hypertension and smoker status. Blood samples of approximately 10 ml were collected by venipuncture of the cubital vein; the venipuncture procedure was conducted by NHS staff as a regular procedure for pre-operative tests. The timing of the preoperative blood samples was the same for the retrospective and prospective study (0900). The samples were analysed for coagulation profile, C-reactive protein, full blood count, HbA1C, kidney function and lipid profile. For the benefit of the coagulation profile, the blood was collected into a blue bottle (Figure 10), as these contain buffered sodium citrate, which reversibly bind to calcium ions in the blood; this disrupts the clotting cascade. Transfusion blood products are also treated with sodium citrate preserving the blood in an uncoagulated state.

Figure 10. Blue bottle used for haematological tests

Yellow bottles, as depicted in Figure 11, were used to collect blood for serum analysis of creatinine, electrolytes (sodium and potassium), lipids (TC, HDL and TG) and urea. This bottle is known in the laboratory as the serum separating tube. Yellow bottles contain silica particles that activate clotting and an inert polymer serum separating gel to facilitate easy centrifugal separation of serum.

Patients were seen at 12 weeks to assess for stump healing by the vascular surgeon according to the standard protocol which involved assessment of the stump including the suture line, stump oedema and skin around the stump. This was done at the vascular outpatient clinic in the Royal Infirmary of Edinburgh.

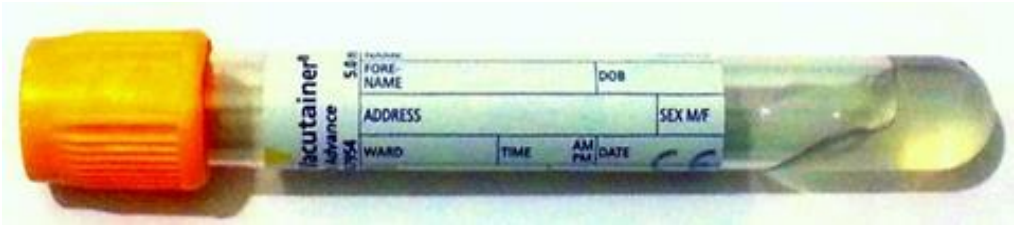


Figure 11. Yellow bottle use for kidney function test and lipid profile

3.9 Statistical analysis

For the prognostic model development, a total of 300 participants were recruited with the resultant demographic data yielding descriptive statistics including both mean and standard deviation. Univariate logistical regression was employed to determine the relationship between independent and dependent variables, noting in particular that reliance on univariate statistical significance may produce indiscrimination through the pre-selection of predictors. As such, in place of depending solely on statistical pre-selection, expert opinion and previous research offers a superior alternative for the first selection of predictors.

From a set of 300 patient's data, the following set of clinically important predictors of stump healing were identified:

Kidney Function Test

- Blood Urea
- Creatinine

Coagulation Test

- Prothrombin Time (PT)
- International Normalization Ratio (INR)

Lipid Profile

- Total Cholesterol (TC)
- Triglyceride (TG)

- Low Density Lipoprotein (LDL)
- High Density Lipoprotein (HDL)

Electrolytes

- Serum Sodium
- Serum Potassium

Inflammatory markers

- White Cell Count (WCC)
- C-reactive protein (CRP)

Glycaemic control

- Glycosylated Haemoglobin (HbA1c)

In addition to these biomedical markers demographic data were analysed.

- Age
- Gender
- Type of Amputation
- Diabetic status
- Hypertension
- Smoking status (ever *versus* never)

Statistical analyses were conducted using SPSS (IBM, SPSS Statistics 20.0) and data are expressed as the mean and 95% Confidence Interval (CI). At first, the model only considered individual predictor and the outcome measure to explore the relationship between these two variables. This was followed by univariate analysis of categorical variables using a Chi square test to establish which of the risk factors differed significantly between stumps that healed and the ones which did not. Univariable logistic regression was used to explore the relationship between the independent variables and stump healing (dependent variable). A logistic regression model neither assumes the linearity in the association between the risk factors and the response variable, nor does it require normally distributed variables. For a variable to be entered into the model, a p value of 0.25 or less was required. Only those predictors with a p-value of 0.25 or less were determined to be relevant and included in the next stage.

Regression model was used for the prediction of stump healing. There are different types of regression modelling.

- Linear Regression (if outcome variable is continuous)
- Multinomial Logistic Regression (if outcome variable is more than two)
- Logistic Regression (if outcome variable is binary or dichotomous)
- Cox regression model (can be used for a “time to event” model)

The relationship between a dichotomous (categorical) dependent variable and dichotomous (metric/categorical) independent variable was analysed by employing logistic regression, since this method, in terms of the independent variables, makes no prior supposition of normality, linearity or homogeneity of variance. As discriminant analysis would require qualification of these assumptions which the data could not satisfy, logistic regression was the superior method of choice.

The equation for simple linear regression is:

$$Y = a + bx + e_i$$

Where y-outcome, a- intercept, b- slope related to x (explanatory variable), e- error term or random noise.

$$Y = logit\ (p) = a + bx + e_i$$

$$\log(\frac{p}{1-p}) = a + bx + e_i$$

The regression equation estimated by logistic regression is given by:

$$\log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots\ldots + \beta_n X_n$$

Where p is the probability of event and effect of independent variable (x) increase or decrease risk of this event. β_0 is the intercept and β_1 till β_n are the regression coefficients.

3.9.1 Performance of prognostic model

A method to assess the fit of a logistic regression model is by comparing the expected and observed numbers of positives for different subgroups of the data. In a robust model, the observed and expected numbers are sufficiently close. The Hosmer-Lemeshow test was used to assess the accuracy of the predictive model by comparing the predicted probabilities against the observed probabilities.

To establish the discriminatory power of the model a Receiver Operating Characteristic Curve (ROC) was generated. This was used to determine the model's effectiveness in differentiating between a healed and non-healed stump. The range of Receiver Operating Characteristic Curve ranges from 0 to 1. A value of 0.5 means that the model has no discrimination power. The

discrimination power increases as the value increases from 0.5 to 1. The larger the Area Under the Curve, the better the diagnostic test. If the area is 1.0, the test is "ideal" because it achieves 100% sensitivity and 100% specificity. If the Area Under the Curve is 0.5, then you have a test that has effectively 50% sensitivity and 50% specificity, which is no better than flipping a coin (Brubaker, 2008). The area under the Receiver Operating Curve for a prognostic model is typically between 0.6 and 0.85 (Royston *et al.* 2009). Sensitivity and specificity analysis were reported. Sensitivity is the proportion of the true positive outcomes (for example, truly diseased subjects) that are predicted to be positive. Specificity is the proportion of the true negative outcomes (for example, truly disease-free subjects) that are predicted to be negative.

3.9.2 Validation of the developed model

As Vergouwe *et al.* (2005) indicated, predictive logistic regression models are important tools to provide estimates of patient outcome probabilities. Hence model validation is the most important step of developing a prediction rule. For this purpose, 100 participants were recruited.

In general, there are two forms of validation. The first type of validation also called internal validation is performed in the context of an individual study, for example, by splitting the study data set into one data set to build the model (development set) and one data set to test performance (test set, also called the validation set). The appealing feature of internal validation is its convenience, as it does not require collection of data beyond the original study. The second form of validation which is the external validation utilises a different data set provided by a different study circumvents these issues. Validation on heterogeneous external data sets allows for evaluation of the generalizability of the risk prediction tool to wider populations than originally reported. In this study, the latter approach was taken for validation.

A total of 100 participants were recruited for the model validation phase. A model precisely predicting probabilities for patients in the retrospective data would not guarantee accurate predictions for new patients from related populations, for example patients treated not long ago or patients from a different centre, therefore, the performance of prognostic models needed to be verified in the newly treated patient group (external validation) (Harrell *et al.* 1996). External validation was performed on those data obtained from an independent set of consecutive patients who had undergone vascular access surgery using the final development model. Predicted probabilities for individual patients in the validation set were calculated. Model discrimination was assessed by Receiver Operating Characteristic Curve analysis. Evaluation of calibration is important if model predictions are used for making clinical decisions. A calibration plot formed by the Hosmer-Lemeshow test, which illustrates how the observed and expected proportions compare, assessed the calibration of the final model for stump healing.

3.9.3 Data protection

Throughout the duration of the study, the information collected from patients was securely stored in a safe place in the vascular department of the Royal Infirmary of Edinburgh and accessibility was restricted to research team members only. Each patient was issued with a unique identification number, which corresponded to his or her personal details, information and consent forms. This same number was used in all stages of the study to ensure confidentiality and to protect identifying personal details. Access to the link anonymised data collected by the principal investigator throughout the study was only available to the key investigators and associated collaborators. The procedure detailed above maintained the duty of confidence to the participants throughout the study. Collected data was stored on a password-protected laptop and back-up discs. Data storage and subsequent destruction was in accordance

with the Data Protection Act 1998. Patients' confidentiality was paramount during the collection of the events and the subsequent submission of manuscripts for publication. The data files included only basic demographic data such as participant number, age, sex etc. Written documentation and data were stored in a paper format in the participants' medical notes as per normal clinical practice. As per the health care records policy, these records will be destroyed after 5 years following discharge.

3.9.4 Indemnity

All participants were informed about the procedure followed during the study. They were also informed about any possible harm they might suffer and how it would be addressed. If patients had any concern about any aspect of this study, they were given the contact details of the principal researcher. They were also provided with the principal investigator's contact details if they had any questions. Alternatively, they could also contact the independent advisor who was aware about the project but was not directly involved in this research (contact details given) or the NHS complaints team, if they wished to complain formally. Further information could be viewed in Appendix VIII.

3.10 Validity and reliability

3.10.1 Measurement of blood pressure

Measuring blood pressure (BP) in the arm (brachial artery) provides an accurate representation of the corresponding pressure in the aorta. The BP was measured using sphygmomanometer with a stethoscope with a standard procedure. The measurement of the blood pressure was carried out after the participants had relaxed for ten minutes in a room with an average room temperature of 20-25-degree Celsius temperature, the cuff of the sphygmomanometer being positioned on the left arm of the participants, 2-3 cm above the cephalic vein. For an accurate

measurement, the participants were requested to position their arm on a flat surface in such a way that the cuff was aligned to the heart; additionally, the size of the cuff was checked to ensure that it was adequate for each participant, as shown below:

- Small Cuff (16-24cm)
- Medium Cuff (24 - 36cm)
- Large Cuff (26 - 45cm)
- Extra Large Cuff (42 - 60cm)

In accordance with Beevers *et al.* (2001) and Perloff *et al.* (1993), the procedure was explained to the participants in order to provide reassurance in the event that their blood pressure was initially high.

CHAPTER 4

RESULTS

4.1 Retrospective study analysis

4.1.1 Descriptive statistics of the retrospective group

A total of three hundred patient's data who underwent a major lower extremity amputation operation between the years 2006 and 2009 at the Royal Infirmary of Edinburgh were identified after a search of the vascular unit electronic patient record (Proton® and Apex® software) at the Royal Infirmary of Edinburgh. Two hundred and fourteen (71.3%) of the 300 identified patients were male with 86 being females (28.7%).

Ages of the participants ranged from 34 to 97 years, with a mean age of 71.16 ± 14.5 years. Percentage of patients found to have diabetes mellitus was 46.0% (n=138) hypertension 94.3% (n=283) and 81.7% (n=245) were smokers. Percentage of patients who underwent an above knee amputation was 53.7% (n=161) with 46.3% (n=139) undergoing a below knee amputation. Seventy one percent (n=214) were noted to be males. Percentage of patients found to have an abnormal serum potassium and abnormal serum sodium were 16% (n=48) and 10.7% (n=32) respectively. Twenty two percent (n=68) and 48.7% (n=125) of patients were found to have abnormal serum creatinine and serum urea respectively. Among the subjects 71.3% (n=62) were noted to have poor diabetes control. Fifty nine percent (n=179) had abnormal white cell count. Healing of the stump as defined was achieved in sixty three percent (n=189) of patients. General distribution of independent variables is shown in Table 3.

Table 3: Characteristics of independent predictive variables for lower limb stump healing in the retrospective group

Clinical characteristics	(n=300)	% Total
Gender		
Male	214	71.3
Female	86	28.7
Amputation		
AKA	161	53.7
BKA	139	46.3
Age		
≤50 yrs.	13	4.3
>50 yrs.	287	95.7
diabetes mellitus		
No	162	54
Yes	138	46
Hypertension(mm/Hg)		
No	17	5.7
Yes	283	94.3
Smoker		
No	55	18.3
Yes	245	81.7
K^+ (3.6-5 mmol/L)		
Abnormal	48	16
Normal	252	84
Na^+ (135-145 mmol/L)		
Abnormal	32	10.7
Normal	268	89.3
Creatinine (60-120 µmol/L)		
Abnormal	68	22.7
Normal	232	77.3
Urea (2.5-6.6 mmol/L)		
Abnormal	125	48.7
Normal	175	58.3
CRP		
≤ 5	15	5.1
> 5	282	94.9
WCC (4-11x10/l)		
Abnormal	179	59.5
Normal	121	40.5
PT (seconds)		
≤13.5	173	57.7
>13.5	127	42.3
INR		
≤1.2	206	68.7

>1.2	94	31.3
TC (mmol/L)		
≤ 5	249	84.4
>5	46	15.6
TG (mmol/L)		
≤ 2.1	239	82.4
>2.1	51	17.6
HDL(mmol/L)		
≤ 1.1	178	61.6
>1.1	112	38.4
HbA1c (%)		
≤6.5	5	5.7
6.5-7.5	20	23.0
>7.5	62	71.3

Table 3: Independent patient factors and blood markers distribution in the subgroups. Data values are expressed as number and percentage (%). n- number of participant, PVD-Peripheral Vascular Disease, diabetes mellitus-diabetes mellitus, HTN-Hypertension, CRP- C reactive protein, WCC- white cell count, K-Potassium, Na-Sodium, PT-Prothrombin time, INR-International normalization ratio, TC- Total Cholesterol, TG- Triglyceride, HDL- High density lipoprotein. The normal ranges for the blood markers were adopted from the RIE laboratory protocols as mentioned in the appendix.

4.1.2 Association between gender and stump healing in the retrospective group

Two hundred and fourteen (71.3%) of the total patients (300) were men, and eighty six (28.7%) women. The figure 12 shows the association between stump healing and gender in patients who underwent lower limb amputation. Out of two hundred and fourteen male patients, one hundred and forty one had a healed lower limb stump (65.9%) and seventy three patients' stumps failed to heal (34.1%). Male gender was noted to be associated with stump healing (p=0.001*). Among the female patients, forty eight patients had healed their stumps out of a total of eight six (55.8%) and failure to heal was noted in 44.4% (n=38). No significant (p=0.281) difference was observed between the healed and non-healed stump among the female gender. The table below shows the gender wise distribution of the number of patients who healed their stumps.

Stump healing was not found to be associated (p=0.102) with male and female gender (Table 4).

Table 4: Gender and stump healing in the retrospective group

	Healed	Not Healed	Total	Chi-Square	*P* value
Female	48	32	86		
Male	141	73	214	2.671	0.102
Total	189	111	300		

Table 4: The distribution and association between stump healing (healed or not healed) and gender (male or female). p=0.102 indicating that stump healing was independent of the gender.

Figure 12: Stump healing in males and females in the retrospective group

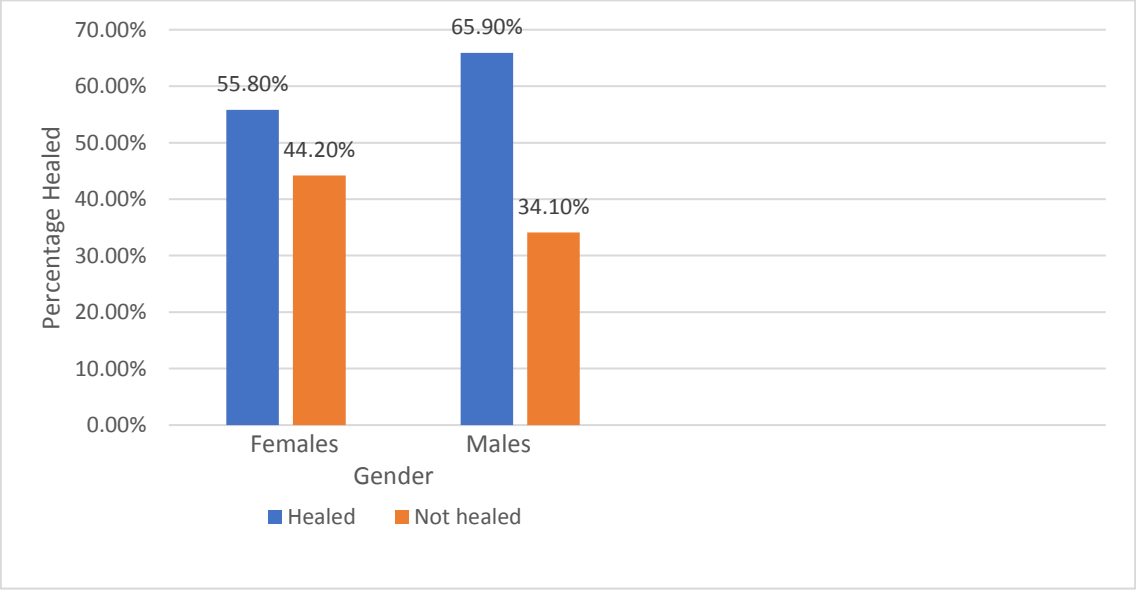


Figure 12 showing association between success and failure of stump healing among male and female gender who underwent lower limb amputation. Numbers written on top of the bar indicate the percentage. p value for males and female gender were p=0.001* and p=0.281 respectively.

4.1.3 Association between type of amputation and stump healing in the retrospective group

Two types of lower limb amputation were considered, that is, above knee and below knee. The figure 13 shows the association between stump healing and the type of amputation in patients who underwent lower limb amputation. Out of three hundred patients, one hundred and sixty one (53.7%) had an above knee amputation among which healing was seen in 68.3% (n=110). A significant (p=0.001*) difference was observed between healed and non-healed stump among the above knee amputation group. The total numbers of below knee amputations were one hundred and thirty nine (46.3%) out of which seventy nine healed (56.8%) (Figure 13). No significant (p=0.107) difference was observed between stump healing and below knee amputation. The table shows distribution of the number of patients who healed their stumps based on the type of amputation. Stump healing was found to be associated (p=0.040*) with type of amputation (Table 5).

Table 5: Type of amputation and stump healing in the retrospective group

Type of Amputation	Healed	Not Healed	Total	Chi-Square	*P* value
AKA	110	51	161		
BKA	79	60	139	4.224	0.040*
Total	189	111	300		

Table 5 shows the distribution and association between stump healing (healed or not healed) and type of amputation (above knee or below knee). p=0.040* indicating that stump healing was dependent on the type of amputation.

Figure 13: Stump healing and the types of amputation in the retrospective group

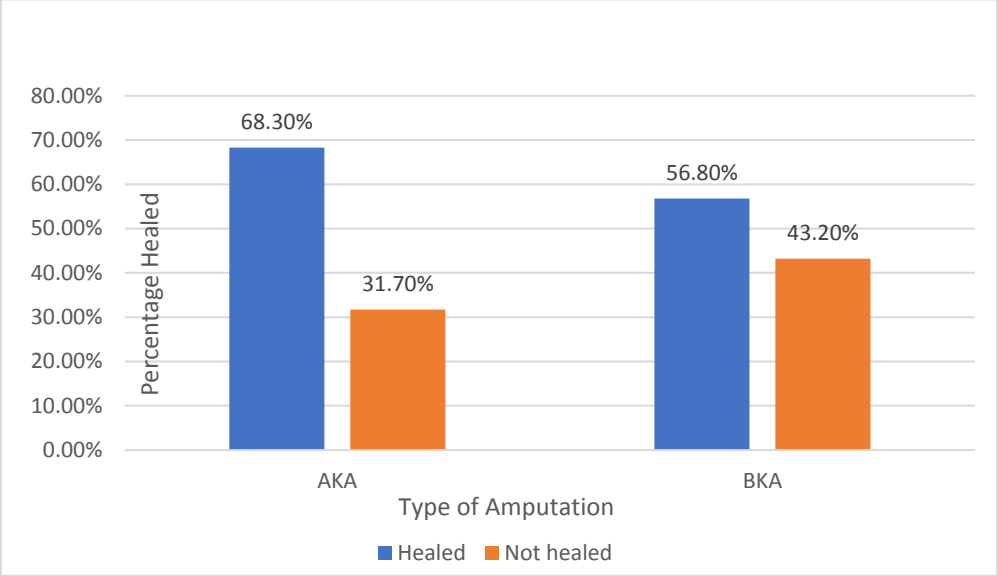


Figure 13 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p value for above and below knee amputation were p=0.001* and p=0.107 respectively.

4.1.4 Association between age and stump healing in the retrospective group

The figure 14 shows the association between stump healing and age in patients who underwent lower limb amputation. Of the 300 patients included in the study, two hundred and eighty seven were above the age of 50 years (95.7%) and thirteen below the age of 50 years (4.3%). The youngest patient was aged 34 years and the oldest, 97 years the average age being 71.16. Out of 287 patients above the age of 50 years, 179 healed their stump (62.4%) and 108 patient's stumps failed to heal (37.6%) (Figure 14). A significant difference was observed between stump healing and patients above the age of 50 years (p=0.001*) but not the group below the age of 50 years (p=0.052). The table shows an age wise distribution of the number of patients who healed their stumps. The healing of the stump was independent of the age (p=0.288) (Table 6).

Table 6: Age and stump healing in the retrospective group

Age	Healed	Not Healed	Total	Chi-Square	*P* value
≤ 50	10	3	13		
> 50	179	108	287	1.130	0.288
Total	189	111	300		

Table 6 shows the distribution and association between stump healing (healed or not healed) and type of amputation (above knee or below knee). *p*=0.288 indicating that stump healing was independent of age.

Figure 14: Stump healing and age in the retrospective group

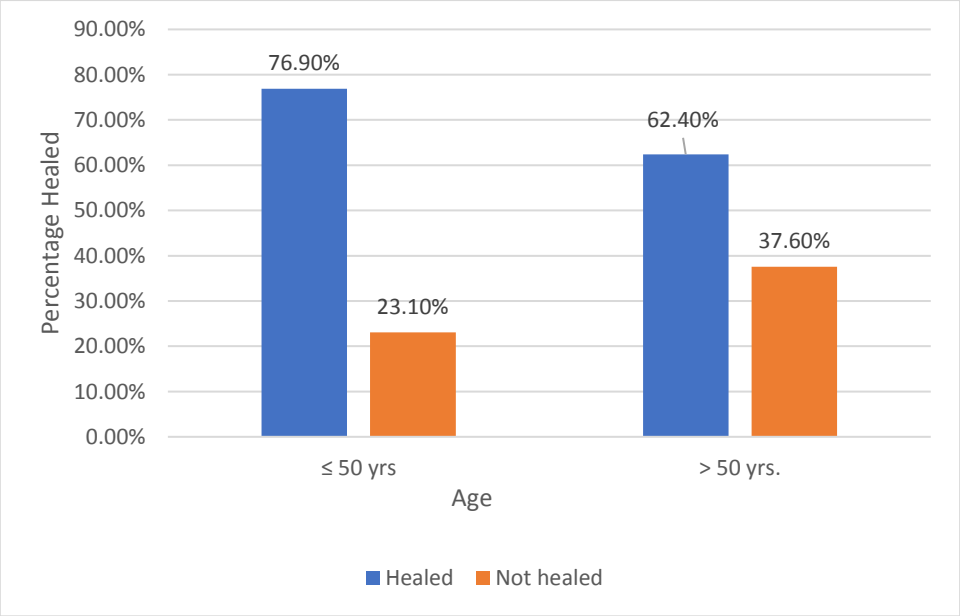


Figure 14 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p value for patients with above and below the age of 50 years were *p*=0.001* and *p*=0.052 respectively.

4.1.5 Association between diabetes mellitus and stump healing in the retrospective group

The figure 15 shows the association between stump healing and diabetes mellitus in patients who underwent lower limb amputation. Out of the 300 included patients, there were one hundred and thirty eight in the diabetes mellitus cohort and one hundred and sixty two in the non-diabetes mellitus cohort. Among the diabetic population, 85 (61.6%) healed and 53 (38.4%) failed to heal their stump. In the non-diabetics, 104 healed their stump (64.2%) in comparison to 58 (35.8%) who failed to heal their stump (Figure 15). A significant difference was observed between stump healing and both diabetic (p=0.006*) and non-diabetic patients (p=0.001*). The table below shows the distribution of the number of patients who healed their stumps in the diabetic and the non-diabetic group. However, stump healing was independent of diabetes mellitus (p=0.642) (Table 7).

Table 7: Diabetes mellitus and stump healing in the retrospective group

Diabetes mellitus	Healed	Not Healed	Total	Chi-Square	*P* value
No	104	58	162		
Yes	85	53	138	0.217	0.642
Total	189	111	300		

Table 7 shows the distribution and association between stump healing (healed or not healed) and type of amputation (above knee or below knee). p=0.642 indicating that stump healing was independent of diabetes.

Figure 15: Stump healing in patients with diabetes mellitus in the retrospective group

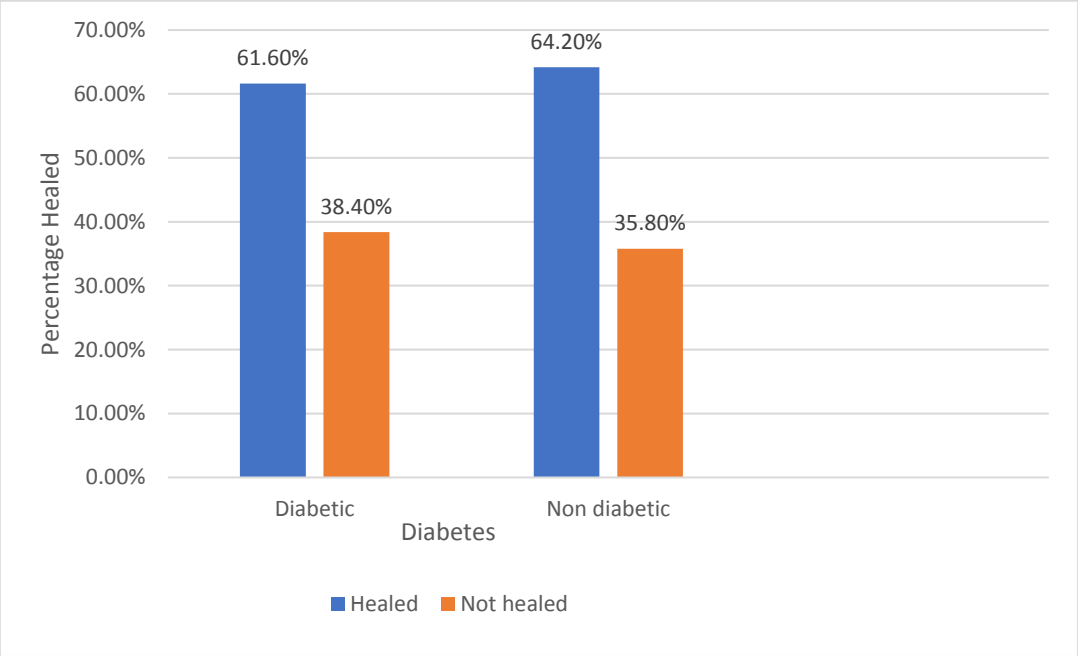


Figure 15 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. The p value for patients with diabetes and without diabetes were (p = 0.006*) and (p = 0.001*) respectively.

4.1.6 Association between hypertension and stump healing in the retrospective group

A total of two hundred and eighty three patients were hypertensive and seventeen were non hypertensive among 300 patients who underwent major lower limp amputation surgery. The figure below shows the association between stump healing and hypertension in patients who underwent lower limb amputation. In the hypertensive group the stump healing rate was 61.8% (n=175) while the failure rate was 38.2% (n=108). On the other hand, 82.4 % (n=14) of the non-hypertensive healed their stump in comparison to 17.6% (n=3) whose stump did not heal (Figure 16). A significant difference was observed between stump healing and patients with (p=0.001*) and without hypertension (p=0.008*). The table shows a distribution of the number

of patients who healed their stumps in the hypertensive and the non-hypertensive group. Overall stump healing was independent of the classification of blood pressure (p=0.089) (Table 8)

Table 8: Hypertension and stump healing in the retrospective group

HTN	Healed	Not Healed	Total	Chi-Square	*P* value
No	14	3	17		
Yes	175	108	283	2.896	0.089
Total	189	111	300		

Table 8 shows the distribution and association between stump healing (healed or not healed) and type of amputation (above knee or below knee). p=0.089 indicating that stump healing was independent of hypertension.

Figure 16: Stump healing and hypertension in the retrospective group

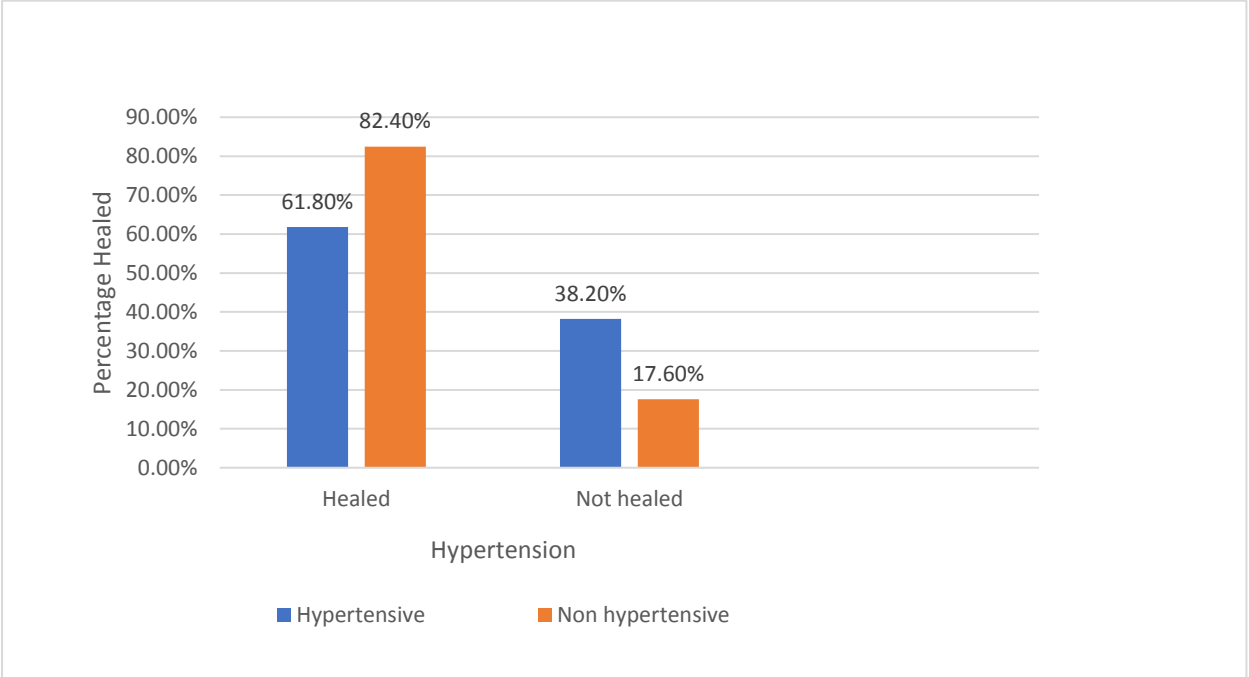


Figure 16 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p value for patients with and without hypertension was (p=0.001*) and (p=0.008*) respectively.

4.1.7 Association between smoking and stump healing in the retrospective group

The impact of smoking was analyzed for all patients (300) enrolled in the study. The figure below shows the association between stump healing and smoking in patients who underwent lower limb amputation. Among 300 patients 245 were smokers and 54 were non-smokers. About Sixty percent (60.8%) (n=149) achieved stump healing and 39.2% (n=96) failed to heal their stump. The success rate of stump healing was 74.1% (n=40) and 25.9% (n=14) failed to heal their stump among nonsmokers (Figure 17). A significant difference was observed between stump healing and both smokers (p=0.001*) and non-smokers (p=0.001*). The table below shows the distribution of the number of patients who healed their stumps among the smoking and non-smoking groups. Stump healing, however, was independent of the smoking (p=0.080) (Table 9).

Table 9: Smoking and stump healing in the retrospective group

Smoking	Healed	Not Healed	Total	Chi-Square	*P* value
No	40	15	55		
Yes	149	96	245	5.045	0.080
Total	189	111	300		

Table 9 shows the distribution and association between stump healing (healed or not healed) and type of amputation (above knee or below knee). p=0.080 indicating that stump healing was independent of smoking.

Figure 17: Stump healing and smoking in the retrospective group

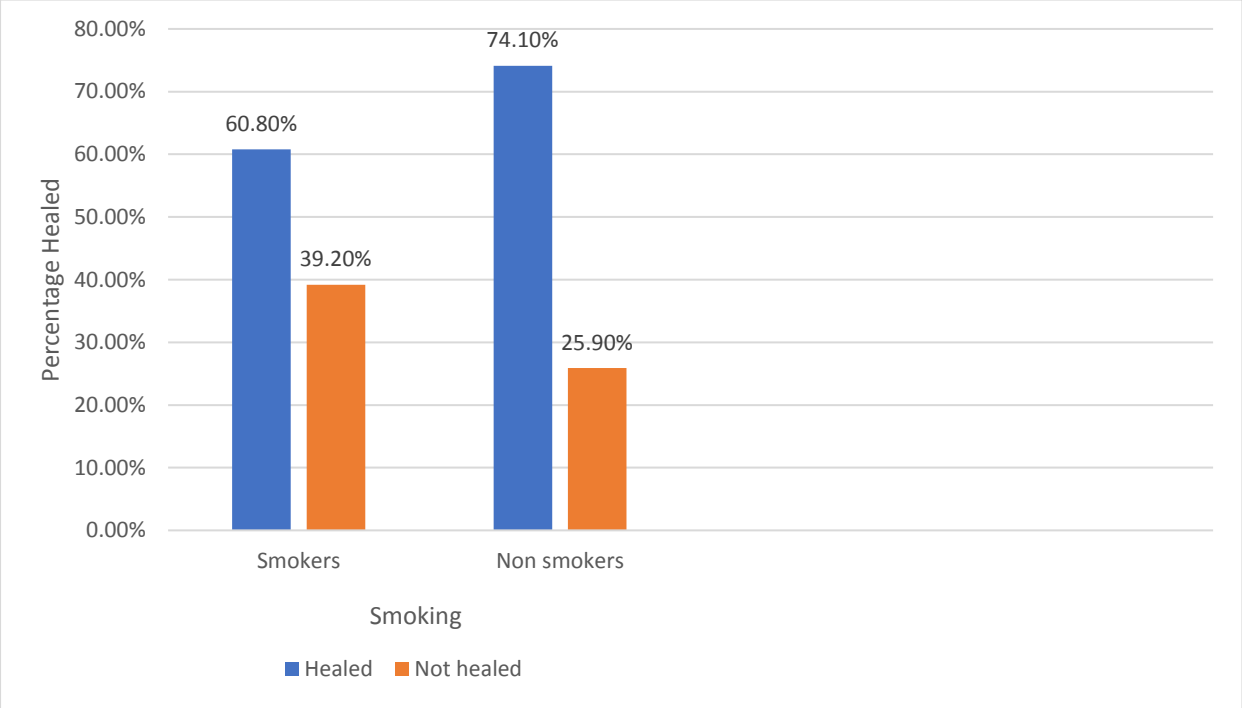


Figure 17 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p value for both smokers and non-smokers was (p=0.001*) and (p=0.001*) respectively.

4.1.8 Association between HbA1c and stump healing in the retrospective group

The impact of the diabetic control was analyzed for the diabetic patients (n=138) enrolled in the study. The figure 18 shows the association between stump healing and HbA1c in patients who underwent lower limb amputation. HbA1c in the last six months was available for eighty seven (63%) patients among the 138 diabetic patients. HbA1c was divided into three categories

namely HbA1c of 6.5% and below, HbA1c between 6.5-7.5% and HbA1c above 7.5%. These groups were based on the patient's diabetic control (optimal, satisfactory and poor) (NICE guidelines, 2015). Eighty percent of the patients (n=4) achieved stump healing among the group with optimal control. The success rate of stump healing was 60% (n=12) and 62.9% (n=39) healed their stump among the satisfactory and poor control group. No significant difference was observed between stump healing and all the three groups; (p=0.324) for the optimal group, (p=0.061) for the satisfactory group and (p=0.052) for the poor control group. The table below shows the distribution of the number of patients who healed their stumps based on their HbA1c. Stump healing was independent of diabetic control (p=706) (Table 10).

Table 10: HbA1c and stump healing in the retrospective group

HbA1c	**Healed**	**Not Healed**	**Total**	**Chi-Square**	***P* value**
≤6.5	4	1	5		
6.5-7.5	12	8	20	0.697	0.706
≥7.5	39	23	62		
Total	55	32	87		

Table 10 shows the distribution and association between stump healing (healed or not healed) and type of amputation (above knee or below knee). p=0.706 indicating that stump healing was independent of diabetic control.

Figure 18: Stump healing in different HbA1c groups in the retrospective group

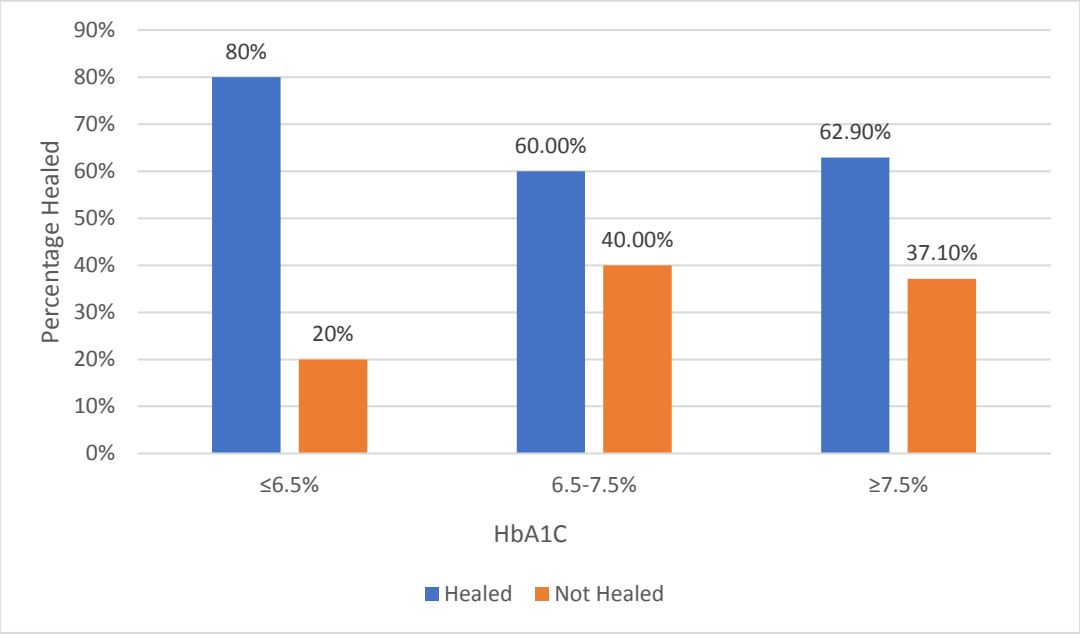


Figure 18 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p value for the different groups were p=0.324 for the optimal group, p=0.061 for the satisfactory group and p=0.052 for the poor control group

4.1.9 Association between serum sodium and stump healing in the retrospective group

The impact of serum sodium on stump healing was analyzed for all patients (300) enrolled in the study. The figure below shows the association between stump healing and serum sodium in patients who underwent lower limb amputation. Among 300 patients, 268 had an abnormal serum sodium and 32 patients had a normal sodium. About sixty three percent (63.4%) (n=170) achieved stump healing and 36.6% (n=98) failed to heal their stump (Figure 19). Stump healing, however, was independent of the classification of serum sodium (p=0.653) (Table 11).

Table 11: Serum sodium and stump healing in the retrospective group

Sodium	Healed	Not Healed	Total	Chi-Square	*P* value
Normal	19	13	32		
Abnormal	170	98	268	0.202	0.653
Total	189	111	300		

Table 11 shows the distribution and association between stump healing (healed or not healed) and serum sodium. *p*=0.653 indicating that stump healing was independent of classification of serum sodium.

Figure 19: Serum sodium and stump healing in the retrospective group

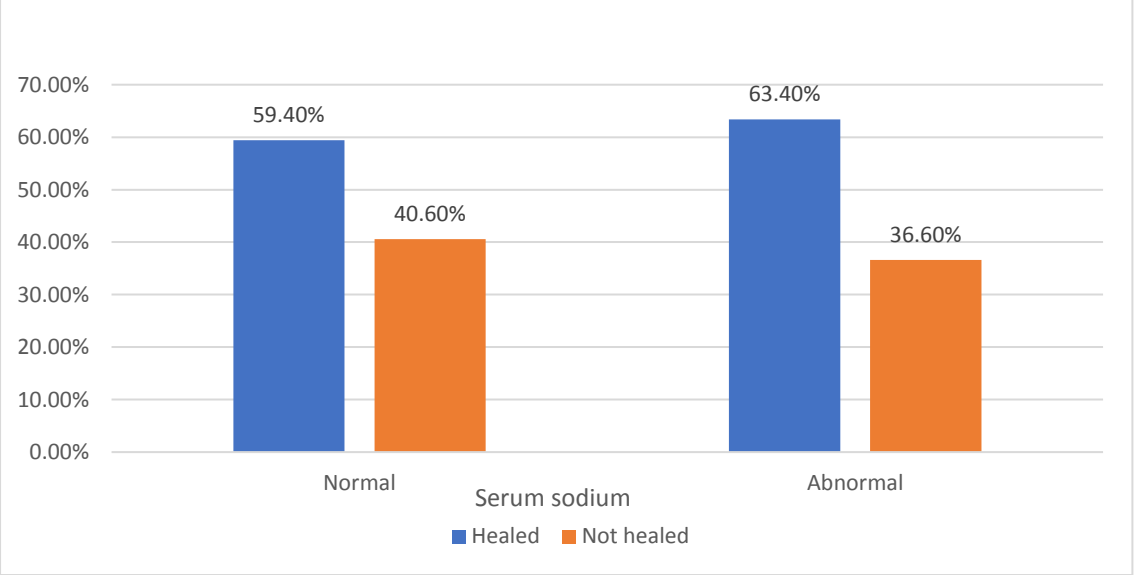


Figure 19 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage.

4.1.10 Association between serum potassium and stump healing in the retrospective group

A total of two hundred and fifty two patients had a normal serum potassium among 300 patients who underwent major lower limp amputation surgery. The Figure 20 below shows the association between stump healing and serum potassium in patients who underwent lower limb

amputation. In the patients with a normal serum potassium the stump healing rate was 62.7% (n=158) while the failure rate was 37.3% (n=94) (Figure 20). The table shows a distribution of the number of patients who healed their stumps in both the groups. Overall stump healing was independent of the classification of serum potassium (*p*=0.804) (Table 12)

Table 12: Serum potassium and stump healing in the retrospective group

Potassium	Healed	Not Healed	Total	Chi-Square	*P* value
Normal	158	94	252		
Abnormal	31	17	48	0.061	0.804
Total	189	111	300		

Table 12 shows the distribution and association between stump healing (healed or not healed) and serum potassium. *p*=0.804 indicating that stump healing was independent of classification of serum potassium.

Figure 20: Serum potassium and stump healing in the retrospective group

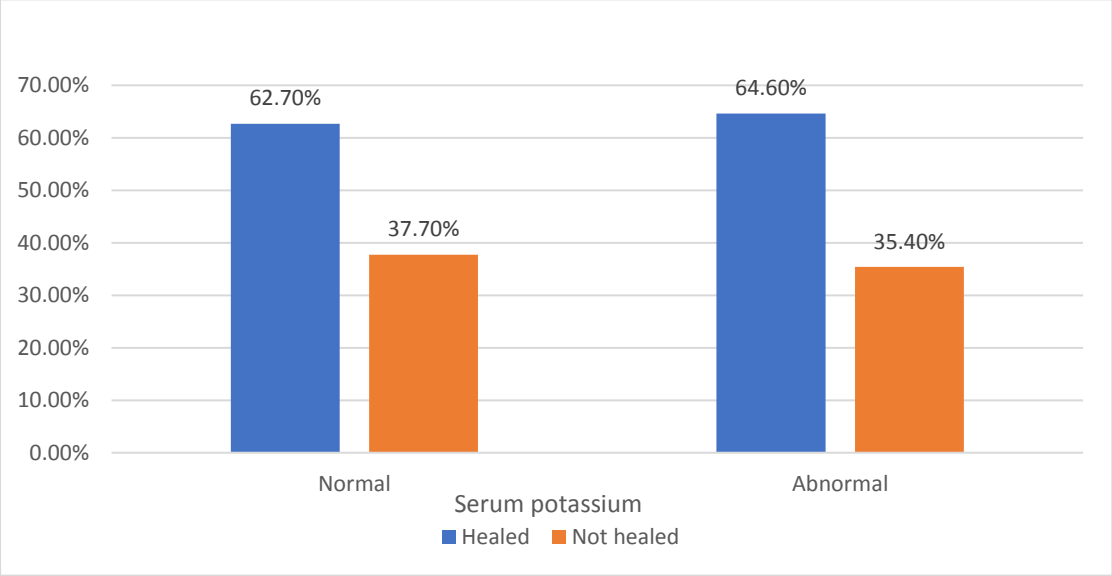


Figure 20 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage.

4.1.11 Association between serum creatinine and stump healing in the retrospective group

The impact of serum creatinine on stump healing was analyzed for all patients (300) enrolled in the study. The Figure 21 below shows the association between stump healing and serum creatinine in patients who underwent lower limb amputation. Among 300 patients, 232 had an abnormal serum creatinine and 68 patients had a normal creatinine. About sixty five percent (65.5%) (n=170) achieved stump healing and 34.5% (n=80) failed to heal their stump (Figure 21). Stump healing, however, was independent of the classification of serum creatinine (p=0.095) (Table 13).

Table 13: Serum creatinine and stump healing in the retrospective group

Creatinine	Healed	Not Healed	Total	Chi-Square	*P* value
Normal	152	80	232		
Abnormal	37	31	68	2.782	0.095
Total	189	111	300		

Table 13 shows the distribution and association between stump healing (healed or not healed) and serum creatinine. p=0.095 indicating that stump healing was independent of serum creatinine.

Figure 21: Serum creatinine and stump healing in the retrospective group

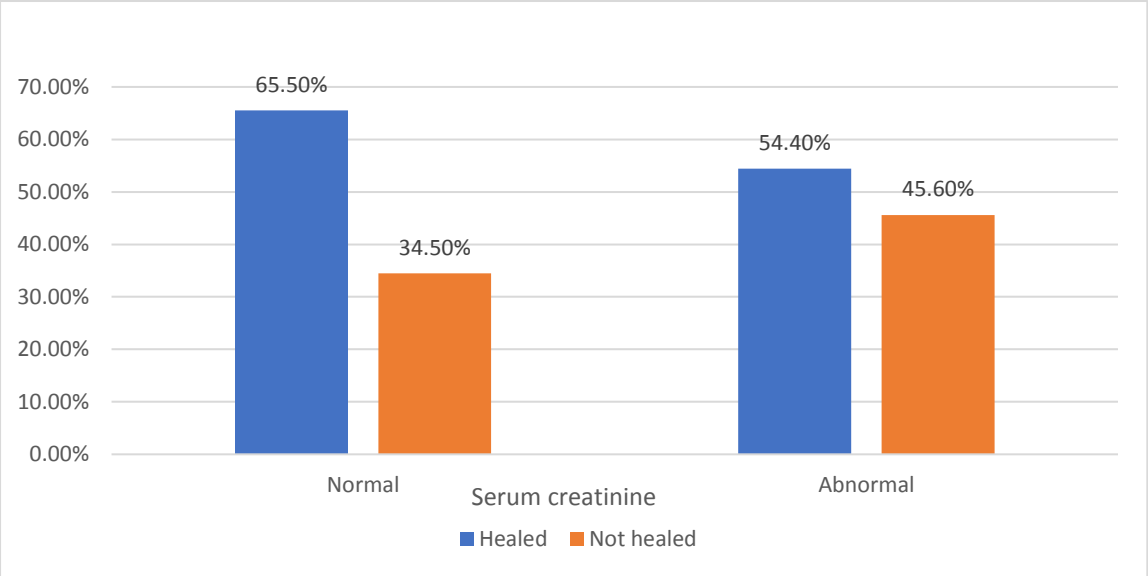


Figure 21 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage.

4.1.12 Association between urea and stump healing in the retrospective group

A total of one hundred and seventy five patients had a normal urea among 300 patients who underwent major lower limp amputation surgery. The Figure 22 below shows the association between stump healing and urea in patients who underwent lower limb amputation. In the patients with a normal urea the stump healing rate was 62.3% (n=109) while the failure rate was 37.7% (n=66) (Figure 22). The table shows a distribution of the number of patients who healed their stumps in both the groups (normal and abnormal urea). Overall stump healing was independent of the classification of urea (p=0.762) (Table 14)

Table 14: Urea and stump healing in the retrospective group

Urea	Healed	Not Healed	Total	Chi-Square	*P* value
Normal	109	66	175		
Abnormal	80	45	125	0.092	0.762
Total	189	111	300		

Table 14 shows the distribution and association between urea, p=0.762 indicating that stump healing was independent of classification of urea.

Figure 22: Urea and stump healing in the retrospective group

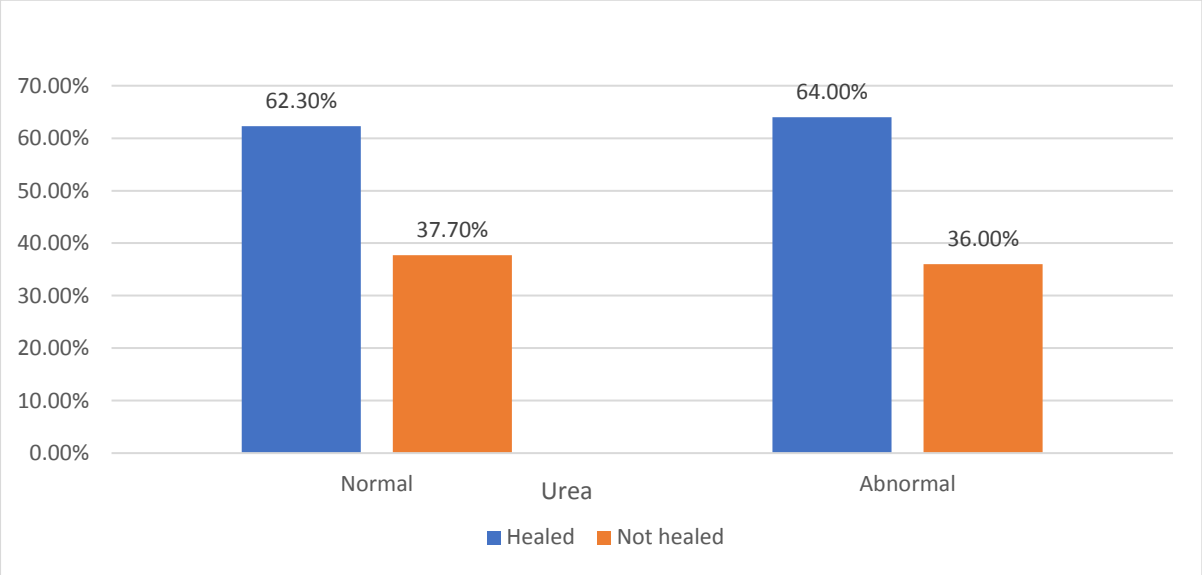


Figure 22 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage.

4.1.13 Association between C-reactive protein and stump healing in the retrospective group

The figure shows the association between stump healing and C-reactive protein in patients who underwent lower limb amputation (Figure 23). The table shows a distribution of the number of

patients who healed their stumps in both the groups (normal and abnormal CRP). The healing of the stump was independent of classification of CRP (p=0.829) (Table 15).

Table 15: C-reactive protein and stump healing in the retrospective group

CRP	Healed	Not Healed	Total	Chi-Square	*P* value
Normal	9	6	15		
Abnormal	177	105	282	0.047	0.829
Total	189	111	300		

Figure 23: C-reactive protein and stump healing in the retrospective group

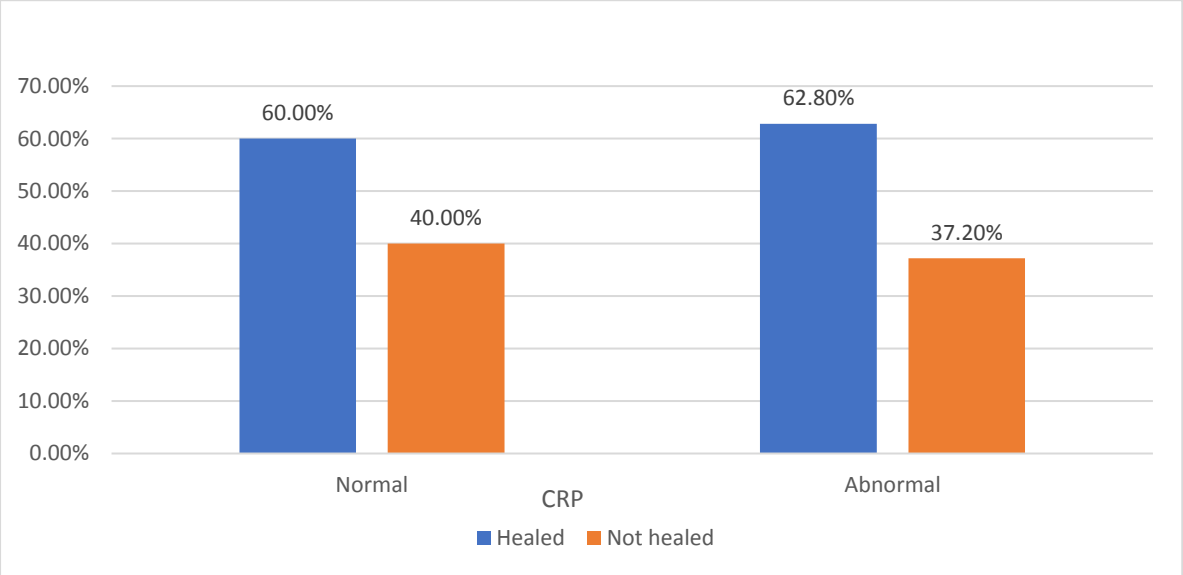


Figure 23 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage.

4.1.14 Association between white cell count and stump healing in the retrospective group

The figure 24 shows the association between stump healing and white cell count in patients who underwent lower limb amputation. The table shows a distribution of the number of patients who healed their stumps in both the groups (with and without normal white cell count). Overall stump healing was independent of the classification of white cell count (p=0.900) (Table 16)

Table 16: White cell count and stump healing in the retrospective group

WCC	Healed	Not Healed	Total	Chi-Square	*P* value
Normal	77	44	121		
Abnormal	112	66	179	0.016	0.900
Total	189	111	300		

Table 16 shows the distribution and association between stump healing (healed or not healed) and white cell count, p=0.900 indicating that stump healing was independent of classification of white cell count.

Figure 24: White cell count and stump healing in the retrospective group

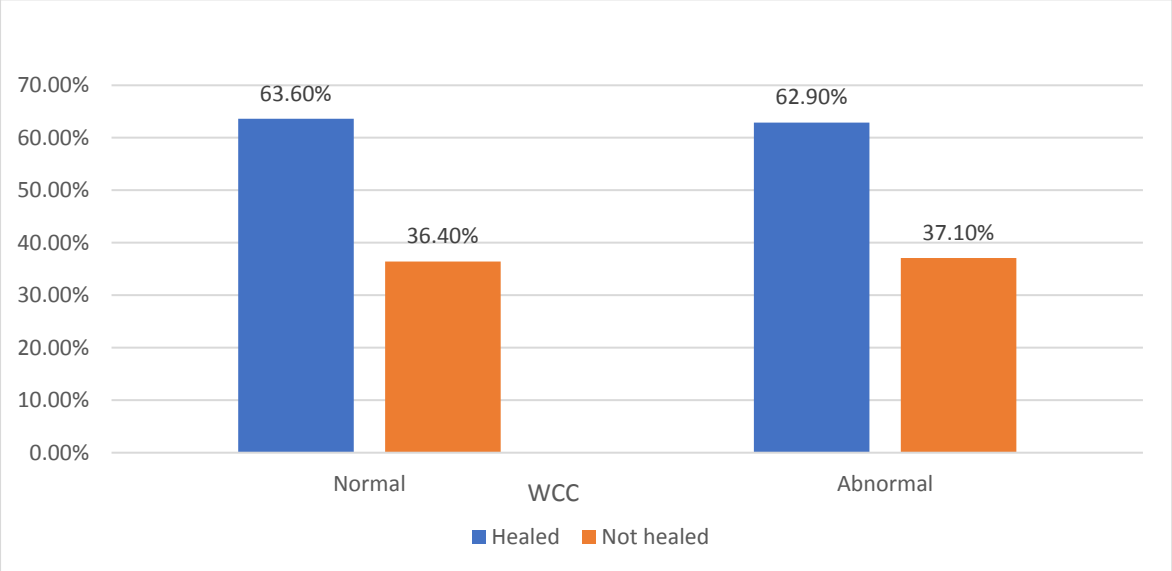


Figure 24 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage

4.1.15 Association between prothrombin time and stump healing in the retrospective group

The figure 25 shows the association between stump healing and prothrombin time in patients who underwent lower limb amputation. Out of the 300 included patients, there were one hundred and seventy three patients with normal PT and one hundred and twenty seven with abnormal PT. The table below shows the distribution of the number of patients who healed their stumps in both the groups. However, stump healing was independent of the classification of PT (p=0.811) (Table 17).

Table 17: Prothrombin time and stump healing in the retrospective group

PT	Healed	Not Healed	Total	Chi-Square	*P* value
Normal	108	65	173		
Abnormal	81	46	127	0.057	0.811
Total	189	111	300		

Table 17 shows the distribution and association between stump healing (healed or not healed) and PT. p=0.811 indicating that stump healing was independent of the classification of PT.

Figure 25: Prothrombin time and stump healing in the retrospective group

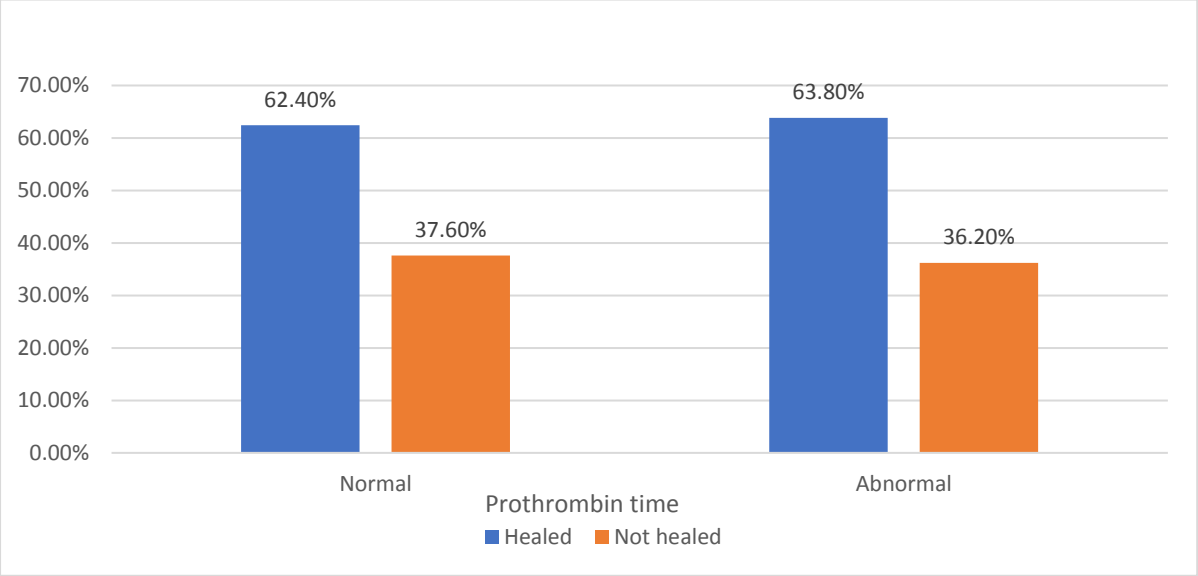


Figure 25 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage

4.1.16 Association between INR and stump healing in the retrospective group

The impact of INR was analyzed for all patients (300) enrolled in the study. The figure 26 below shows the association between stump healing and INR in patients who underwent lower limb amputation. The table below shows the distribution of the number of patients who healed their stumps among both the groups (normal and abnormal INR). Stump healing, however, was independent of the classification of INR (p=0.406) (Table 18).

Table 18: INR and stump healing in the retrospective group

INR	Healed	Not Healed	Total	Chi-Square	*P* value
Normal	133	73	206		
Abnormal	56	38	94	0.689	0.406
Total	189	111	300		

Table 18 shows the distribution and association between stump healing (healed or not healed) and PT, p=0.406 indicating that stump healing was independent of the classification of PT.

Figure 26: INR and stump healing in the retrospective group

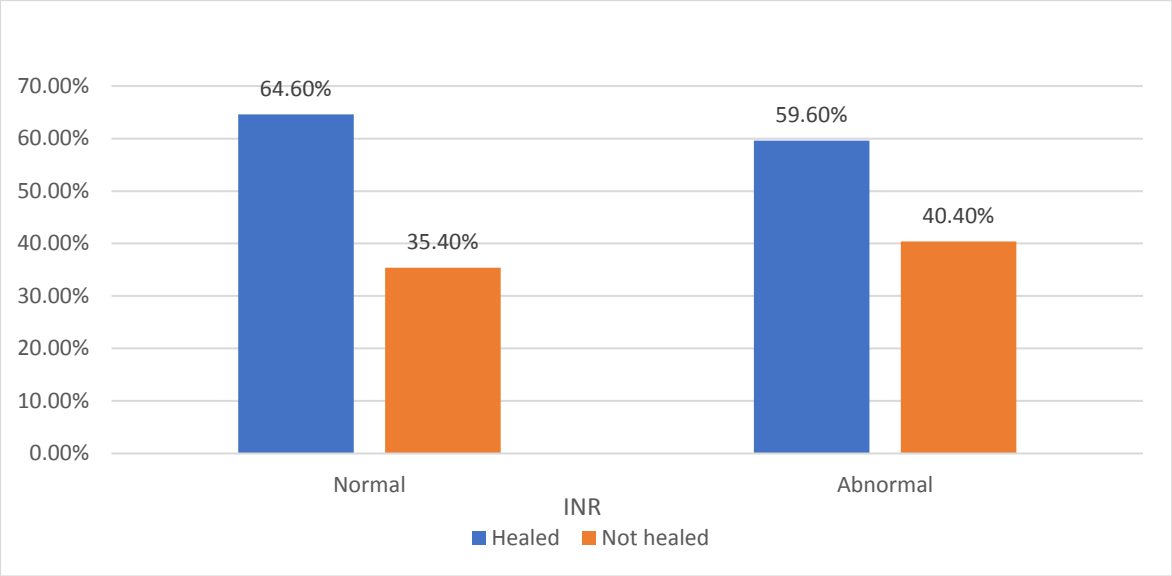


Figure 26 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage

4.1.17 Association between serum total cholesterol and stump healing in the retrospective group

A total of two hundred and forty nine patients had a normal serum total cholesterol among 300 patients who underwent major lower limp amputation surgery. The figure 27 below shows the

association between stump healing and serum total cholesterol in patients who underwent lower limb amputation. The table and the figure 27 shows a distribution of the number of patients who healed their stumps in both he groups. Overall stump healing was independent of classification of total cholesterol (p=0.293) (Table 19) (Figure 27).

Table 19: Serum total cholesterol and stump healing in the retrospective group

TC	Healed	Not Healed	Total	Chi-Square	*P* value
Normal	161	88	249		
Abnormal	26	20	46	1.108	0.293
Total	189	111	300		

Table 19 shows the distribution and association between stump healing (healed or not healed) and serum total cholesterol. p=0.293 indicating that stump healing was independent of the classification of serum total cholesterol.

Figure 27: Serum total cholesterol and stump healing in the retrospective group

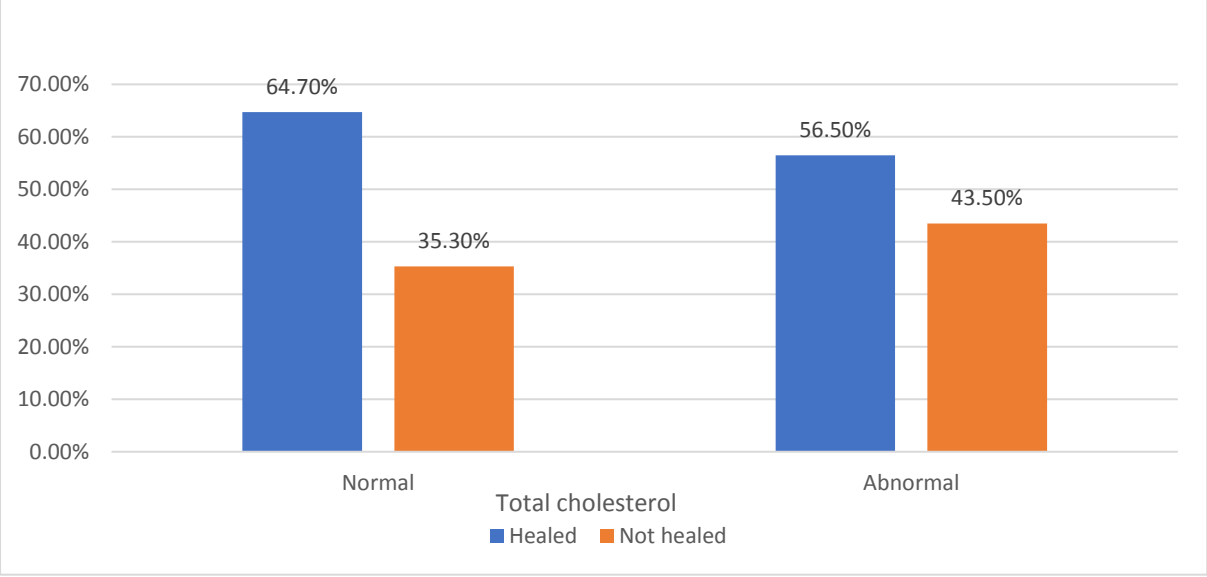


Figure 27 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage

4.1.18 Association between serum triglycerides and stump healing in the retrospective group

The figure 28 shows the association between stump healing and serum triglycerides in patients who underwent lower limb amputation. The table below shows the distribution of the number of patients who healed their stumps in both the groups (with normal and abnormal serum triglycerides). However, stump healing was independent classification of serum triglycerides (p=0.638) (Table 20).

Table 20: Serum triglycerides and stump healing in the retrospective group

TG	Healed	Not Healed	Total	Chi-Square	*P* value
Normal	151	88	239		
Abnormal	34	17	51	0.221	0.638
Total	189	111	300		

Table 20 shows the distribution and association between stump healing (healed or not healed) and serum triglycerides. p=0.642 indicating that stump healing was independent of classification of serum triglycerides.

Figure 28: Serum triglycerides and stump healing in the retrospective group

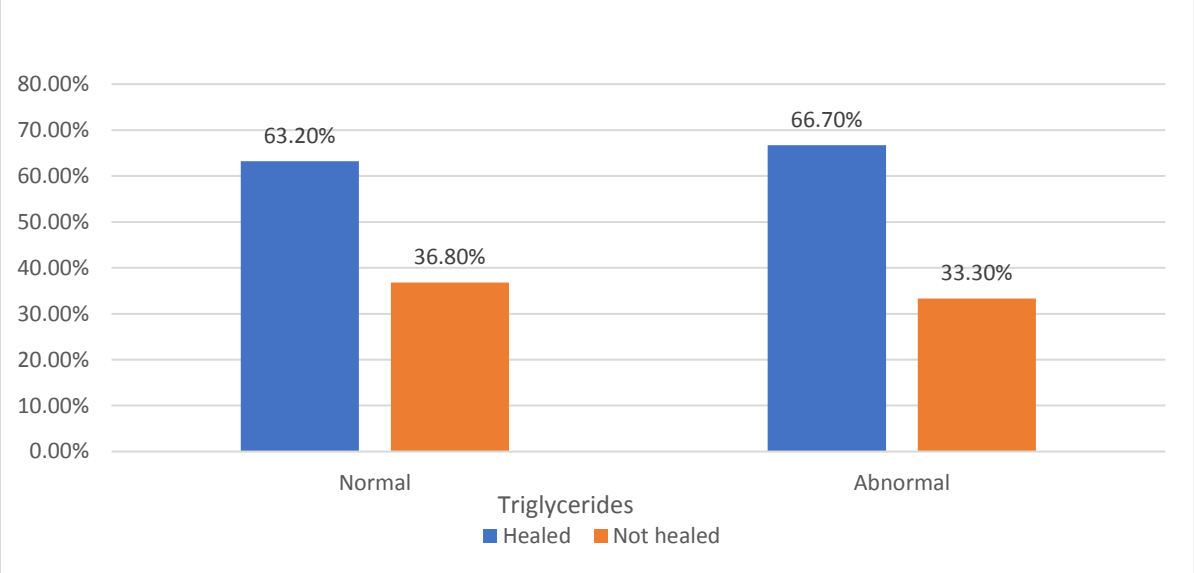


Figure 28 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage

4.1.19 Association between serum high density lipids and stump healing in the retrospective group

The figure 29 shows the association between stump healing and serum HDL in patients who underwent lower limb amputation. The table below shows the distribution of the number of patients who healed their stumps in both the groups (with normal and abnormal serum HDL). However, stump healing was independent classification of serum HDL (p=0.054) (Table 21).

Table 21: Serum high density lipids and stump healing in the retrospective group

HDL	Healed	Not Healed	Total	Chi-Square	*P* value
Normal	121	57	178		
Abnormal	63	48	112	3.721	0.054
Total	189	111	300		

Table 21 shows the distribution and association between stump healing (healed or not healed) and serum HDL. *p*=0.804 indicating that stump healing was independent of classification of serum HDL.

Figure 29: Serum high density lipids and stump healing in the retrospective group

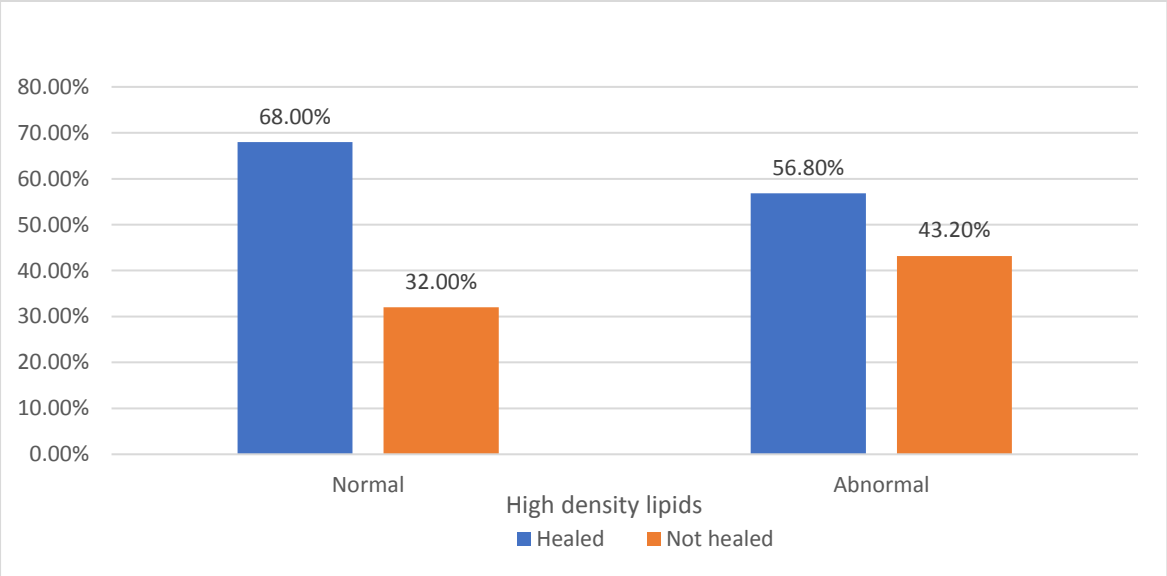


Figure 29 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage

4.2 Descriptive statistics by type of amputation in the retrospective group

4.2.1 Association between gender and stump healing by amputation type

The figure 30 shows the association between stump healing and gender in patients who underwent lower limb amputation. Out of 300, one hundred and thirty nine had a below knee

amputation of which one hundred and five (75.5%) were males and thirty four (24.5%) were females (Table 22). Out of the one hundred and five male patients, sixty patients had a healed lower limb stump (57.1%) and forty five patients stumps failed to heal (42.9%). Of the healed group (n= 79), 75.9% (n=60) were males and 24.1% (n=19) were females. Among the female group, 55.9 % (n=19) stumps had healed and failure to heal was noted in 44.1% (n=15). No significant difference was observed between stump healing and the male (p=0.187) or female gender (p=0.206). Stump healing was not found to be associated (p=0.897) with gender among the below knee amputation group.

Among the above knee amputation group (n=161), one hundred and nine (67.7%) were males and fifty two (32.3%) were females (Table 22). Of the healed group (n= 103), 75.7% (n=78) were males and 24.3% (n=25) were females. Among the male patients, eighty one (74.3%) had a healed lower limb stump while twenty eight (25.7%) patients stumps failed to heal their stump. Among the female group, 55.8% (n=29) stumps had healed and failure to heal was noted in 44.2% (n=23). A significant difference was observed between stump healing and the male (p=0.001*) but not with the female gender (p=0.405). The table below shows the gender wise distribution of the number of patients who healed their stumps. Stump healing was found to be associated (p=0.018*) with gender among the above knee amputation group.

Table 22: Gender and stump healing by amputation type

	Healed		Not Healed		Total		Chi-Square		*P* value	
	AKA	**BKA**	**AKA**	**BKA**	**AKA**	**BKA**	**AKA**	**BKA**	**AKA**	**BKA**
Female	29	19	23	15						
Male	81	60	28	45						
Total	110	79	51	60	161	139	5.593	0.017	0.018*	0.897

Table 22 shows the distribution and association between stump healing (healed or not healed) and gender (male or female) among AKA and BKA.

Figure 30: Stump healing in males and females by amputation type

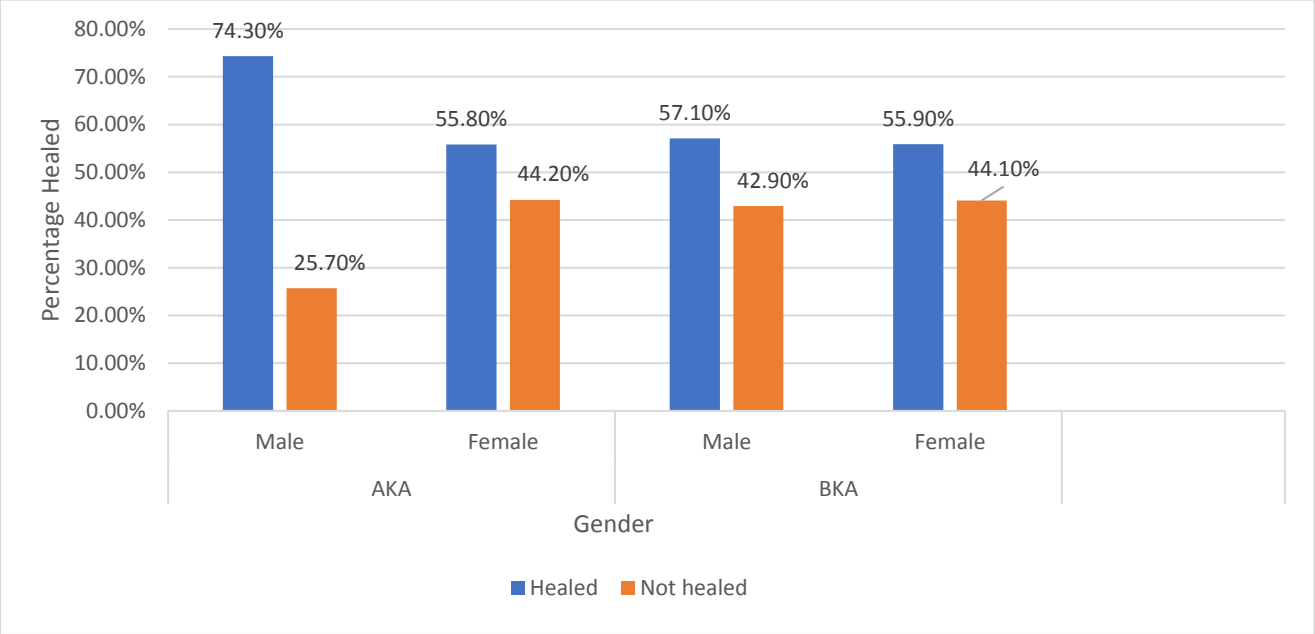


Figure 30 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p=0.018* for the AKA group indicating that stump healing was dependent of the gender while the p=0.897 BKA group indicating there was no relation between gender and stump healing.

4.2.2 Association between age and stump healing by amputation type

The figure 31 shows the association between stump healing and age in patients who underwent lower limb amputation. Of the 136 patients who had a below knee amputation in the retrospective study, 92.8% (n=129) were above the age of 50 years and 7.2% (n=10) below the age of 50 years. The youngest patient was aged 34 years and the oldest, 86 years the average

age being 73.27. Out of 129 patients above the age of 50 years, 72 healed their stump (55.8%) and 57 patient's stumps failed to heal (44.2%) (Figure 31). Among the group who were below 50 years, 30% (n=10) failed to heal their stump. No significant difference was observed between stump healing and both patients below the age of 50 years (p=0.206) and above the age of 50 years (p=0.187). The healing of the stump in the below knee amputation group was independent of the age (p=0.383) (Table 23).

Of the161 patients included in the study who had an AKA, 158 (98.1%) were above the age of 50 years and 3 (1.9%) below the age of 50 years. The youngest patient was aged 34 years and the oldest 97 years, the average age being 73.36. Out of 158 patients above the age of 50 years 67.7% (n=108) healed their stump and 32.3% (n=53) patient's stumps failed to heal (Figure 17). A significant difference was observed between stump healing and both patients above the age of 50 years (p=0.001*). No p value was noted for the group below the age of 50 years. The table shows an age wise distribution of the number of patients who healed their stumps. The healing of the stump was independent of the age (p=0.234) in the above knee amputation group (Table 23).

Table 23: Age and stump healing by amputation type

Age	Healed		Not Healed		Total		Chi-Square		*P* value	
	AKA	BKA	AKA	BKA	AKA	BKA	AKA	BKA	AKA	BKA
≤ 50	1	7	2	3						
> 50	107	72	51	57						
Total	108	79	53	60	161	139	1.417	0.761	0.234	0.383

Table 23 shows the distribution and association between stump healing (healed or not healed) and type of amputation (above knee or below knee).

Figure 31: Stump healing and age by amputation type

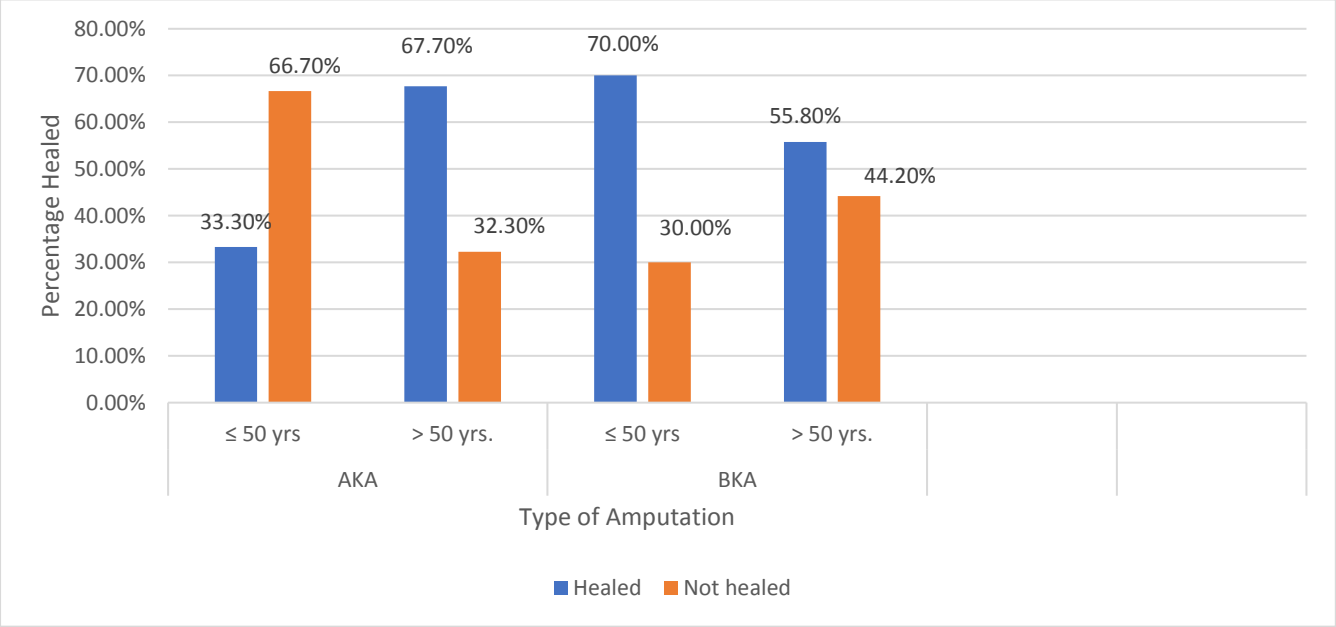


Figure 31 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p=0.234 and p=0.383 for AKA and BKA group indicating that stump healing was independent of age.

4.2.3 Association between diabetes mellitus and stump healing by amputation type

The figure 32 shows the association between stump healing and diabetes mellitus in patients who underwent lower limb amputation. Out of the 139 patients who underwent below knee amputation, there were 78 in the diabetes mellitus cohort and 61 in the non-diabetes mellitus cohort. Among the diabetic population 57.7% (n=45) healed and 42.3% (n=33) failed to heal

their stump. In the non-diabetics, 55.7% (n=34) healed their stump in comparison to 44.3% (n=27) who failed to heal their stump (Figure 32). No significant difference was observed between stump healing and both diabetic (p=0.174) and non-diabetic patients (p=0.370). Stump healing was independent of diabetes mellitus in the below knee amputation group (p=0.817) (Table 24).

Out of AKA group (n=161), there were 60 in the diabetes mellitus cohort and 101 in the non-diabetes mellitus cohort. Among the diabetic population, 66.7% (n=40) healed and 33.3% (n=20) failed to heal their stump. In the non-diabetics, (n=104) healed their stump 69.3% (n=70) in comparison to 30.7% (n=31) who failed to heal their stump (Figure 32). A significant difference was observed between stump healing and both diabetic (p=0.010*) and non-diabetic patients (p =0.001*). The table below shows distribution of the number of patients who healed their stumps among the diabetic and non-diabetic groups. Stump healing was independent of Diabetes (p=0.642) in the above knee amputation group (Table 24).

Table 24: Diabetes mellitus and stump healing by amputation type

Diabetes mellitus	**Healed**		**Not Healed**		**Total**		**Chi-Square**		***P* value**	
	AKA	**BKA**	**AKA**	**BKA**	**AKA**	**BKA**	**AKA**	**BKA**	**AKA**	**BKA**
No	70	34	31	27	86					
Yes	40	45	20	33	214					
Total	110	79	51	60	161	139	0.121	0.53	0.728	0.817

Table 24 shows the distribution and association between stump healing (healed or not healed) and diabetes mellitus.

Figure 32: Stump healing in patients with diabetic mellitus by amputation type

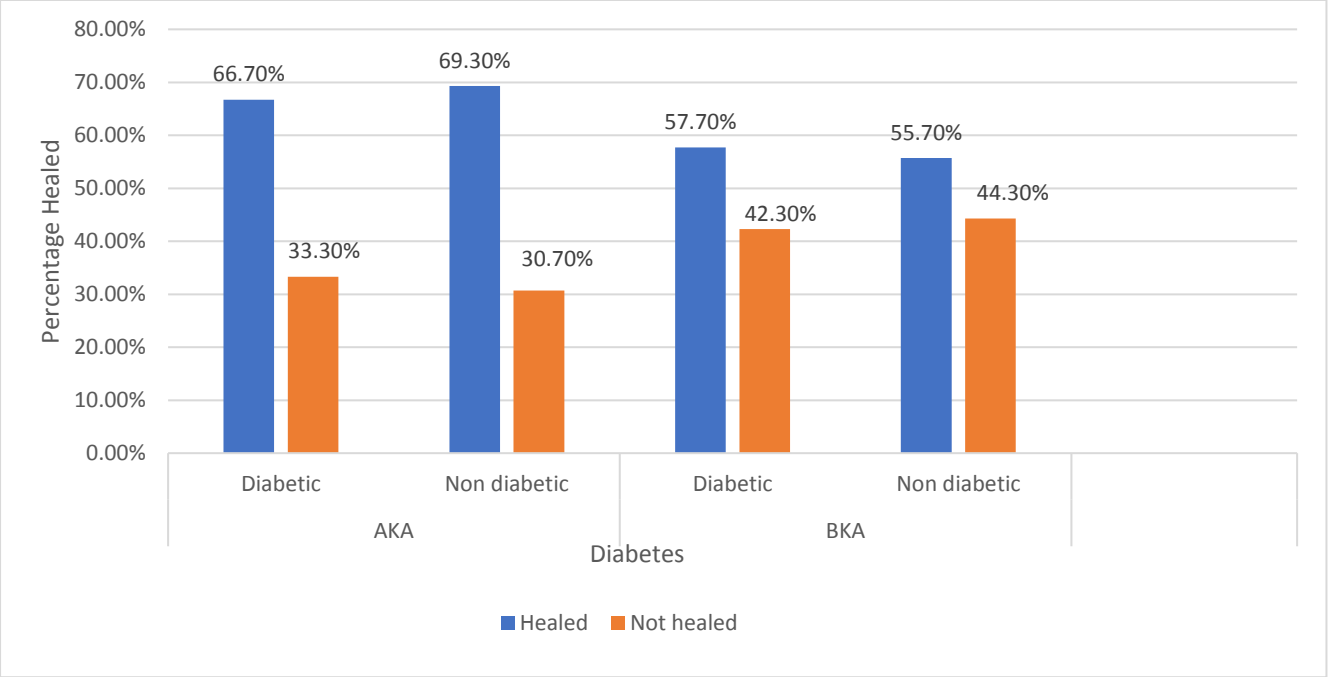


Figure 32 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p=0.728 and p=0.817 for AKA and BKA group indicating that stump healing was independent of diabetes.

4.2.4 Association between hypertension and stump healing by amputation type

The figure 33 shows the association between stump healing and hypertension in patients who underwent lower limb amputation. A total of 136 patients were hypertensive and 3 were non hypertensive among 139 patients who underwent major lower limp amputation surgery. In the hypertensive group the stump healing rate was 56.6% (n=77) while the failure rate was 43.4% (n=59). On the other hand, 66.7 % (n=2) of the non-hypertensive healed their stump in comparison to 33.3% (n=1) whose stump did not heal. No significant (p=0.68) difference was observed between stump healing and patients with (p=0.123) and without hypertension

(p=0.564). Stump healing in the below knee amputation group was independent of the blood pressure (p=0.728) (Table 25)

Among 161 patients who underwent an above knee amputation surgery, 147 were hypertensive and 14 were non hypertensive. In the hypertensive group, the stump healing rate was 66.7% (n=98) while the failure rate was 33.3% (n=49). On the other hand, 85.7 % (n=12) of the non-hypertensive healed their stump in comparison to 14.3% (n=2) who failed to heal. (Figure 33). A significant difference was observed between stump healing and patients with (p=0.001*) and without hypertension (p=0.008*). The table below shows a distribution of the number of patients who healed their stumps in the hypertensive and the non-hypertensive group. Stump healing was independent of the blood pressure in the above amputation group (p=0.143) (Table 25).

Table 25: Hypertension and stump healing by amputation type

HTN	**Healed**		**Not Healed**		**Total**		**Chi-Square**		***P* value**	
	AKA	**BKA**	**AKA**	**BKA**	**AKA**	**BKA**	**AKA**	**BKA**	**AKA**	**BKA**
No	12	2	2	1						
Yes	98	77	49	59						
Total	110	79	51	60	161	139	2.143	0.121	0.143	0.728

Table 25 shows the distribution and association between stump healing (healed or not healed) and hypertension

Figure 33: Stump healing and hypertension by amputation type

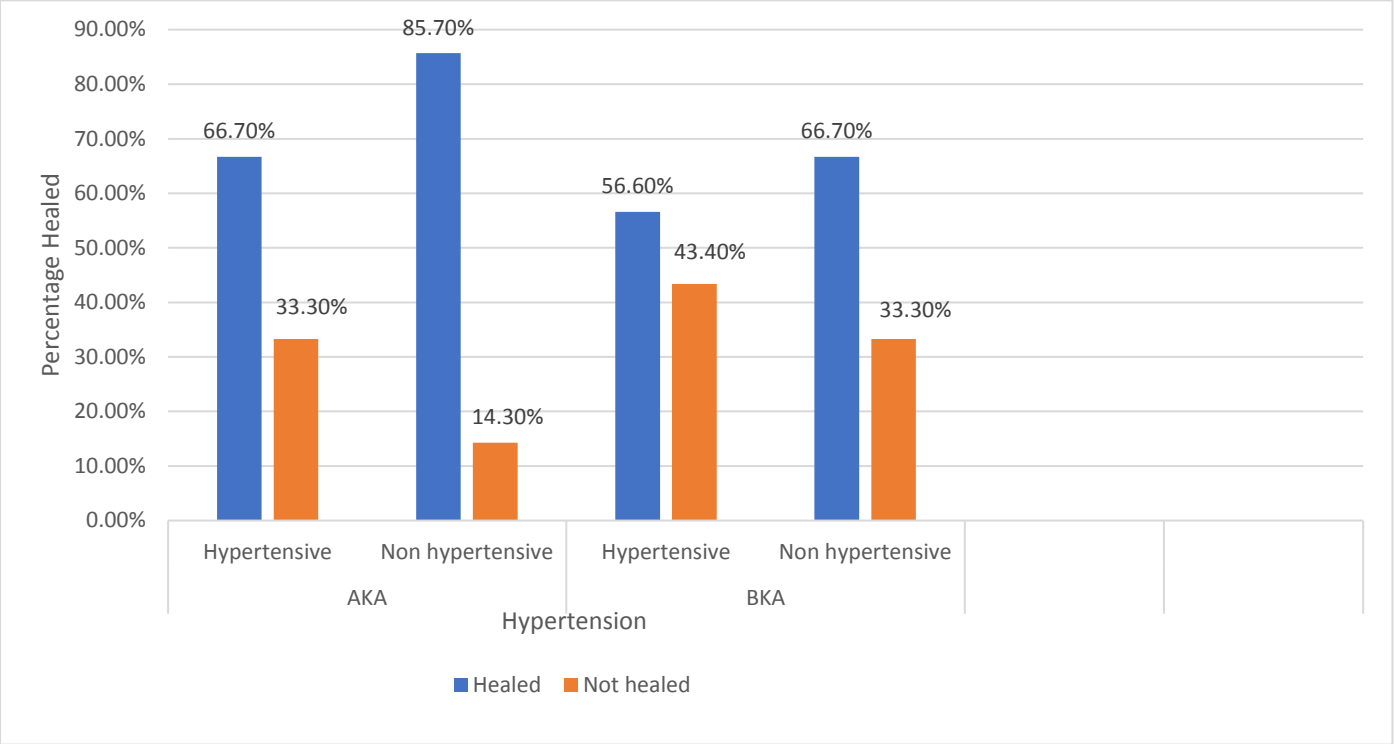


Figure 33 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p=0.143 and p=0.728 for AKA and BKA group indicating that stump healing was independent of hypertension.

4.2.5 Association between smoking and stump healing by amputation type

The figure 34 shows the association between stump healing and smoking in patients who underwent lower limb amputation. Among 139 patients who underwent below knee amputation, 114 were smokers and 25 were non-smokers. Among the smoking group, 54.4% (n=62) achieved stump healing and 45.6% (n=52) failed to heal their stump. The success rate of stump healing was 68% (n=17) and 32% (n=8) failed to heal their stump among nonsmokers (Figure 34). No significant (p=0.68) difference was observed between stump healing and both

smokers (p=0.349) and non-smokers (p=0.072). Stump healing was independent of the smoking (p=0.213) among the below knee amputation group (Table 26).

Among 161 patients who underwent above knee amputation, 132 were smokers and 29 were non-smokers. Among the smoking group, 66.4% (n=88) achieved stump healing and 33.6% (n=44) failed to heal their stump. The success rate of stump healing was 79.3% (n=23) and 20.7% (n=6) failed to heal their stump among nonsmokers (Figure 34). A significant difference was observed between stump healing and both smokers (p=0.020*) and non-smokers (p=0.001*). The table below shows the distribution of the number of patients who healed their stumps among the smoking and non-smoking groups. Stump healing was independent of the smoking in the above knee amputation group (p=0.080) (Table 26).

Table 26: Smoking and stump healing by amputation type

Smoking	Healed		Not Healed		Total		Chi-Square		*P* value	
	AKA	BKA	AKA	BKA	AKA	BKA	AKA	BKA	AKA	BKA
No	23	17	6	8						
Yes	88	62	44	52						
Total	111	79	50	60	161	139	1.839	1.549	0.175	0.213

Table 26 shows the distribution and association between stump healing (healed or not healed) and smoking.

Figure 34: Stump healing and smoking by amputation type

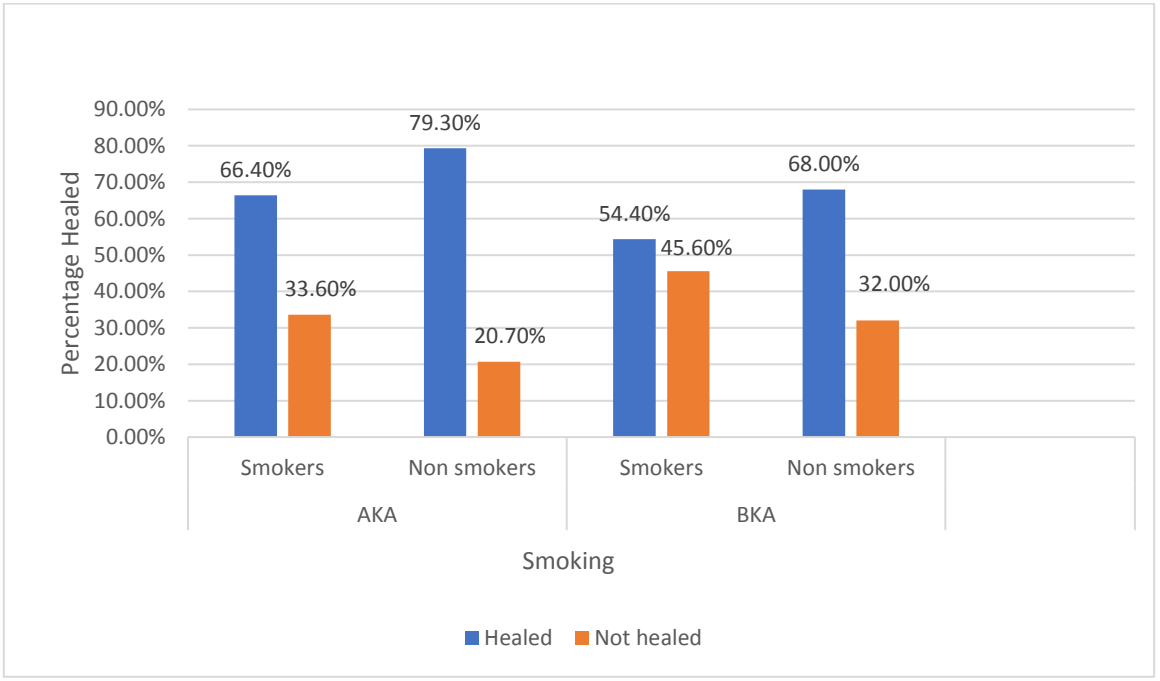


Figure 34 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p=0.175 and p=0.213 for AKA and BKA group indicating that stump healing was independent of smoking.

4.3 Univariate analysis of independent variables

Retrospective analysis of the derivation set of 300 lower limb stumps that were performed between 2005 and 2009 revealed a failure to heal rate of 37% (n = 111). Access characteristics and univariate analysis of clinical variables for the prediction of stump healing are shown in Table 27. Univariate analysis found seven variables to be associated with lower limb stump healing: type of amputation (OR 1.638; 95% CI 1.022-2.627), gender (OR 1.529; 95% CI

0.971-2.549) , hypertension (OR 0.347; 95% CI 0.098-1.236) , smoking (OR 0.347; 95% CI 0.098-1.236), serum sodium (OR 1.711 95% CI 1.039-2.818), serum creatinine (OR 1.592; 95% CI 0.920-2.755) and High Density Lipids (OR 1.617; 95% CI 0.991-2.640). Further variables, namely age (OR 2.01; 95% CI 0.542-7.470), diabetes mellitus (OR 0.894; 95% CI 0.559-1.431), white cell count (OR 1.031; 95% CI 0.639-1.665) and Prothrombin Time (OR 0.994; 95% CI 0.587-1.517) were added to the model secondary to their strong clinical association with the stump healing.

Table 27: Univariate analysis of independent variables to lower limb stump healing

Clinical characteristics	% Stump Healed	Total %	Crude OR (95% CI)	P Value
Age				
≤ 50 yrs.	76.9	4.3	2.011(0.542-7.470)	0.297
> 50 yrs.	62.4	95.7		
Gender				
Male	65.9	71.3	1.529 (0.971-2.549)	0.103*
Female	55.8	28.7		
Type of Amputation				
AKA	68.3	53.7	1.638(1.022-2.627	0.04*
BKA	56.8	46.3		
Diabetes mellitus				
No	64.2	54	0.894(0.559-1.431)	0.642
Yes	61.6	46		
Smoker				
No	74.1	18.3	0.543 (0.281-1.052)	0.191*
Yes	60.8	81.7		
HTN				
No	82.4	5.7	0.347 (0.098-1.236)	0.103*
Yes	61.8	94.3		
K+				
Abnormal	64.6	16	0.922 (0.48-1.756)	0.804
Normal	62.7	84		
Na+				
Abnormal	54.3	10.7	1.711 (1.039-2.818)	0.035*
Normal	67	89.3		
WCC				
Abnormal	62.9	59.5	1.031 (0.639-1.665)	0.901
Normal	63.6	40.5		
CRP				
≤ 5	60	5.1	0.890 (0.308-2.571)	0.829
>5	62.8	94.9		
Creatinine				
≤120	65.5	77.3	1.592(0.920-2.755)	0.097*
>120	54.4	22.7		
Urea				
≤ 6.6	62.3	58.3	0.929 (0.577-1.496)	0.762
> 6.6	64	41.7		
PT				
≤ 13.5	62.4	57.7	0.944 (0.587-1.517)	0.811
> 13.5	63.8	42.3		
INR				
≤ 1.2	64.6	68.7	1.236 (0.789-2.041)	0.407
> 1.2	59.6	31.3		
TC				
≤ 5	64.7	84.4	1.407 (0.743-2.664)	0.294
>5	56.5	15.6		
TG				
≤ 2.1	63.2	82.4	0.858 (0.453-1.625)	0.638
> 2.1	66.7	17.6		
HDL				
≤ 1.1	68	61.6	1.617 (0.991-2.640)	0.054*
> 1.1	56.8	38.4		

Table 27: Independent patient factors and blood markers that underwent in univariate analysis and their association with lower limb stump healing. Data values are expressed as value (%), Odds Ratio (OR), Confidence interval (CI) and level of significance (p). *Is used for significant variables having p value <0.25,*Is used for significant variables, diabetes mellitus-diabetes mellitus, HTN-Hypertension, CRP-C Reactive Protein, WCC- White Cell Count, K-Potassium, Na-Sodium, PT-Prothrombin time, INR- International normalization ratio, TC- Total Cholesterol, TG- Triglyceride, HDL- High density lipoprotein.

4.4 Correlation

Logistic regression models often experience serious multi collinearity problems resulting from strong correlations between independent variables. A significant correlation between variables affects the selection of predictors. A correlation table of all the potential predictors was therefore generated with a value greater than 0.70 suggesting a strong correlation. A correlation coefficient was computed to assess the association between the independent variables. Overall, no significant correlation was found between independent variables as summarized in Table 28.

Table 28: Correlation matrix between independent variables

		Constant	Amp	Gender	HTN	Smoking	Na	HDL	Age	DM	PT	WCC
Step 1	Constant	1.000	-.447	-.090	-.005	-.030	-.230	-.310	-.049	-.285	-.068	-.022
	Amp	-.447	1.000	.104	.067	.029	.014	.021	.146	-.137	_.095	-.075
	Gender	-.090	.104	1.000	.020	-.114	-.055	-.027	-.019	.048	.073	.020
	HTN	.005	.067	.020	1.000	-.096	-.062	-.035	.018	-.119	.006	-.044
	Smoking	-.030	.029	-.114	-.096	1.000	.085	-.002	-.015	-.114	.036	.049
	Na	-.230	.014	-.055	-.062	.085	1.000	.090	.072	.110	-.066	-.036
	HDL	-.310	.021	-.027	-.035	-.002	.090	1.000	.001	-.056	-.078	.029
	Age	-.049	.146	-.019	.018	-.015	-.072	.001	1.000	-.075	-.023	-.036
	DM	-.285	-.137	.048	-.119	-.114	.110	-.056	-.075	1.000	-.113	.019
	PT	-.068	_.095	.073	.006	.036	-.066	-.078	-.023	-.113	1.000	-.012
	WCC	-.022	-.075	.020	-.044	.049	-.036	.029	-.036	.019	-.012	1.000

Table 28: *Is used for significant variables, diabetes mellitus-diabetes mellitus, HTN-Hypertension, CRP-C Reactive Protein, WCC- White Cell Count, K-Potassium, Na-Sodium, PT-Prothrombin time, INR- International normalization ratio, TC- Total Cholesterol, TG- Triglyceride, HDL- High density lipoprotein.

4.5 Multivariable associations

In this study, backward stepwise model selection procedure was used for multivariate associations. In backward regression analysis, the model contains all the predictors and SPSS software systematically removes the largest non-significant p-value term until a subset that consists of entirely statistically significant terms are left. All the selected variables are entered at the same time into the model. With each step the variable with the highest p-values is removed (that is, the variable contributing the least). Then the model is re-run with the remaining variables. This step is repeated until there are no variables left with a *p*-value greater than 0.05. Selection is based on the statistical significance of covariables in the data set under study. For the predictive model for lower limb stump healing, variables whose *p*-value was ≤0.25 were assigned to enter multiple logistic regression. So, type of amputation, gender, hypertension, smoking, serum sodium, serum creatinine and HDL cholesterol along with the variables with strong clinical association namely age, diabetes mellitus, white cell count and Prothrombin Time were added to run backward regression to develop the adjusted odd ratio and scoring (Table 28). Three variables were identified which influenced lower limb stump healing in the multivariable model. The lower limb stump healing was relatively 75% more likely in patients with normal serum sodium compared to that of patients with abnormal serum sodium (OR 1.756; 95% CI 1.048-2.942; p 0.031). Patients with normal serum creatinine were 66% more likely to have their stump healed (OR 1.664; 95% CI 0.94 to 2.946; p 0.046). A normal serum High Density Lipid cholesterol resulted in a 75% more likely chance of

healing compared to those with abnormal serum High Density Lipid cholesterol (OR 1.753; 95% CI 1.061 to 2.895; p <0.026).

A predictor score developed using the regression coefficients of these variables is shown in Table 29. To generate score for each predictor variables, score is assigned by dividing Beta Coefficient to significant error. The overall probability for each patient was analysed by adding the scores of each factor. The following prognostic model was derived by using the above prediction model.

Table 29: Multivariable predictors of lower limb stump healing in the retrospective data

Clinical characteristics	**Adjusted OR**	**95% CI**	**P Value**
Serum Sodium (Normal)	1.756	0.310-0.866	0.031
Serum Creatinine (Normal)	1.664	1.49-5.854	0.046
Serum HDL (Normal)	1.753	2.221-11.315	0.026

Table 29: showing the predictors which played significant role in lower limb stump healing in the multivariate analysis.

The overall risk score for each patient was estimated by summing the scores of each significant independent variable. Using the prediction model, the following prognostic equation was developed:

$$\boldsymbol{Risk\ Score}(-\log \boldsymbol{odds}) = \boldsymbol{\beta_0} + \boldsymbol{\beta_1 X_1} + \boldsymbol{\beta_2 X_2} + \ldots\ldots + \boldsymbol{\beta_n X_n}$$

Where

B_0 is the intercept, β_1 till β_n are the regression coefficients and X_1 to X_n are independent variables.

Risk Score- log odds of failure of stump healing [log/ (1/1- P)] = -1.82 + (0.563 × Se Na) + (0.509 × Se Creatinine) + (0.561 × HDL)

Where

Se Na=serum sodium levels, Se Creatinine=serum creatinine levels and HDL=serum High Density Lipid cholesterol

Where all variables are coded 0 for no or 1 for yes. The value −0.182 is called the intercept and the other numbers are the estimated regression coefficients for the predictors, which indicate their mutually adjusted relative contribution to the outcome risk.

4.5.1 Hosmer-Lemeshow Goodness of Fit Test

It's important to examine the appropriateness of fitted models. This was carried out by using the Hosmer and Lemeshow goodness of fit test. The null hypothesis for this test is that the model fits the data, and the alternative is that the model does not fit. The Hosmer-Lemeshow statistic which tests the null hypothesis and plots difference between observed and predicted data was not significant (p>0.87).

Receiver Operating Characteristic Area

The Receiver Operating Characteristic curve area was studied as the indicator of a model performance which suggests how well a parameter can distinguish between two predictive outcomes. The Receiver Operating Characteristic curve, which is defined as a plot of test sensitivity as the y coordinate versus its 1-specificity or false positive rate as the x

coordinate, is an effective method of evaluating the performance of prognostic model. Sensitivity and specificity, which are defined as the number of true positive decisions/the number of actual positive cases and the number of true negative decisions/the number of actually negative cases, respectively, constitute the basic measures of performance of diagnostic tests (Park *et al.* 2004) (Table 30).

Table 30: The decision matrix

Test Results	**Positive**	**Negative**	**Total**
Positive	TP	FP	TP/(TP+FP)
Negative	FN	TN	TN/(FN+TN)
Total	TP/(TP+FN)	TN/(FP+TN)	

Table 30: Where TP: true positive = test positive in actually positive cases, FP: false positive = test positive in actually negative cases, FN: false negative = test negative in actually positive cases, TN: true negative = test negative in actually negative cases. Sensitivity and Specificity of a Test are Defined as TP/ (TP+FN) and TN/ (FP+TN) respectively. Positive predictive value and Positive predictive value are defined as TP/ (TP+FP) and TN/ (FN+TN) respectively.

Receiver Operating Characteristic curve figures are two-dimensional figures in which true positive rate is plotted on the Y axis and false positive rate is plotted on the X axis. In this study Receiver Operating Characteristic curve was constructed by calculating the sensitivity and specificity for consecutive cut-off points according to the predicted probabilities from the logistic regression models. The green line in the figure is the slope of the tangent line at a cut point which gives the likelihood ratio (LR) for that value of the

test. The blue line is the curve showing the result of the study. The closer the curve follows the left-hand border and then the top border of the ROC space, the more accurate the test.

The closer the curve comes to the 45-degree diagonal of the ROC space, the less accurate the test. The area under the Receiver Operating Characteristic curve for prediction of stump healing was 0.612 (95% bias-corrected CI: 0.546 - 0.679), which indicates good model discrimination (Figure 35, Table 31). For binary outcomes, C-index is equal to the area under the ROC curve; C-index varies between 0.5 and 1.0 for sensible models; the greater the value, the better the performance of prognostic model (Miller *et al.* 1993; Harrell *et al.* 1996). Table 31 shows the cut-off score for prediction of stump healing was 0.621 (sensitivity 15.2%, specificity 91.3%, PPV 50% and NPV 65%).

Figure 35: Receiver Operating Curve analysis for prognostic model performance

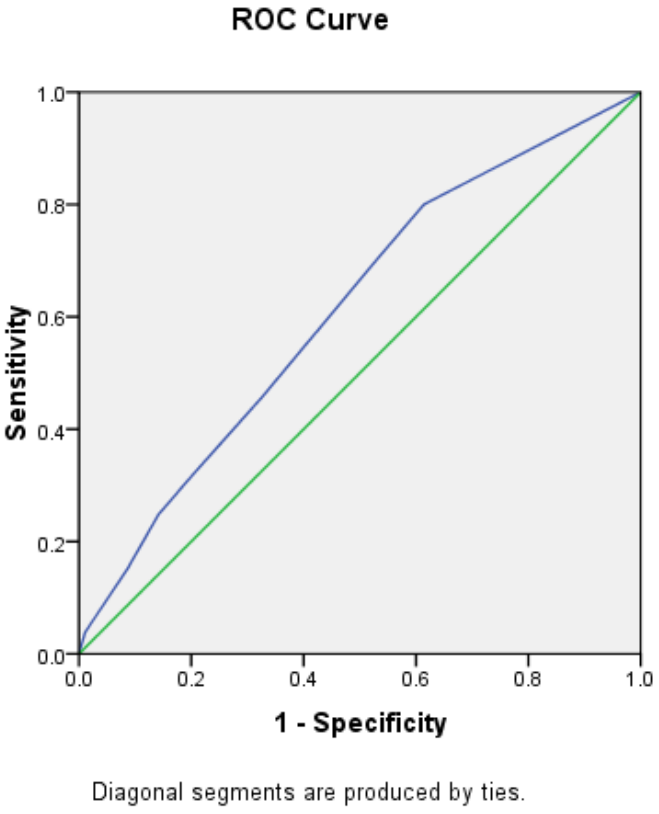


Figure 35 showing Receiver operating characteristics of stump healing. Area under the curve was 0.612 (95% CI: 0.546-0.679), indicating good discriminatory ability of Stump healing.

Table 31: Specificity and sensitivity of the model

Area under the Curve	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
0.612	15.2%	91.3%	50..5%	65.8%

Table 31 showing the area under the ROC is 0.612, which is indicating 61% ability to discriminate between patients with stump healing.

4.6 Validation of prognostic model

Clinical and demographic characteristics of patients in the development and validation sets are shown in Table 32. Patients in the development and validation set were age of 71.16 ± 14.5 and 68±15.5 respectively (p=0.55). The significant difference between the development and validation set was evaluated against p<0.05. Baseline exploration of patients' characteristic discovered the four variables to be significantly different in both development and validation cohort. Patients in the development set compared with the patients in the validation set were more frequently male. Mean of serum creatinine, serum High Density Lipid cholesterol and C reactive protein was statistically different in development and validation set. Overall both cohorts' participant characteristic were similar.

Table 32: Summary of the baseline characteristic of independent variables in the prospective group

Characteristic	Development Set	Validation Set	*p*-Value
Age (≤50/>50)	4.3/95.7	3/97	0.55
Gender (Male/Female)	71.3/28.7	61/39	0.054
Amputation(AKA/BKA)	53.7/46.3	53/47	0.90
diabetes mellitus (Yes/No)	46/54	44/56	0.72
HTN (Yes/No)	94.3/5.7	98/2	0.13
Smoker (Yes/No)	81.7/18.3	85/15	0.39
Urea, mean ±SD (mmol/L)	7.5±5.5	6.6±4.1	0.11
Creatinine, mean ±SD (µmol/L)	100.6±70.4	82.5±43.1	0.003*
K+, mean ±SD (mmol/L)	4.2±0.59	4.1±0.46	0.09
Na+, mean ±SD (mmol/L)	136.3±4.1	135±3.9	0.47
CRP, mean ±SD (mmol/L)	111.3±94.6	90.9±74.6	0.03*
WCC, mean ±SD (mmol/L)	12.8±5.3	11.7±4.2	0.66
PT, mean ±SD (second)	14.3±6.4	14.1±5.0	0.81
INR, mean ±SD (ratio)	1.2±0.5	1.2±0.4	0.23
TC, mean ±SD (mmol/L)	3.9±1.1	3.9±1.1	0.88
TG, mean ±SD (mmol/L)	1.6±0.8	1.5±0.7	0.21
HDL, mean ±SD (mmol/L)	1.1±0.4	1.3±0.8	0.01*
LDL, mean ±SD (mm)	1.9±0.7	1.7±0.8	0.81

Table 32: *Is used for significant difference between two cohorts' p value <0.05, ±SD, Standard Deviation, diabetes mellitus-diabetes mellitus, HTN-Hypertension, PVD-Peripheral Vascular Disease, K-Potassium, Na-Sodium, CRP-C-Reactive Protein, PT-Prothrombin Time, INR- International Normalization Ratio, TC- Total Cholesterol, TG-Triglyceride, HDL- High Density Lipoprotein.

4.7 Prospective study analysis

4.7.1 Descriptive statistics for the prospective group

4.7.2 Association between gender and stump healing in the prospective group

The figure 36 shows the association between stump healing and gender in patients who underwent lower limb amputation. Sixty one (61%) of the total patients (n=100) were men, and thirty nine (39%) women (Figure 36). Of the two sixty one males, thirty seven (60.7%) had a healed lower limb stump and twenty seven (39.3 %) failed to heal their stumps. No significant (p=0.159) difference was observed between stump healing and the male gender. Among the female patients, 48.7 % (n=19) stumps had healed and failure to heal was noted in 51.3% (n=20). No significant (p=0.873) difference was observed between healed and non-healed stump among the female gender. The table below shows a gender wise distribution of the number of patients who healed their stumps. Stump healing was not found to be associated (p=0.241) with male and female gender (Table 33).

Table 33: Gender and stump healing in the prospective group

	Healed	**Not Healed**	**Total**	**Chi-Square**	***P* value**
Female	19	20	39		
Male	37	24	61	1.376	0.241
Total	56	44	100		

Table 33 shows the distribution and association between stump healing (healed or not healed) and gender (male or female). p=0. 241 indicating that stump healing was independent of the gender.

Figure 36: Stump healing and gender in the prospective group

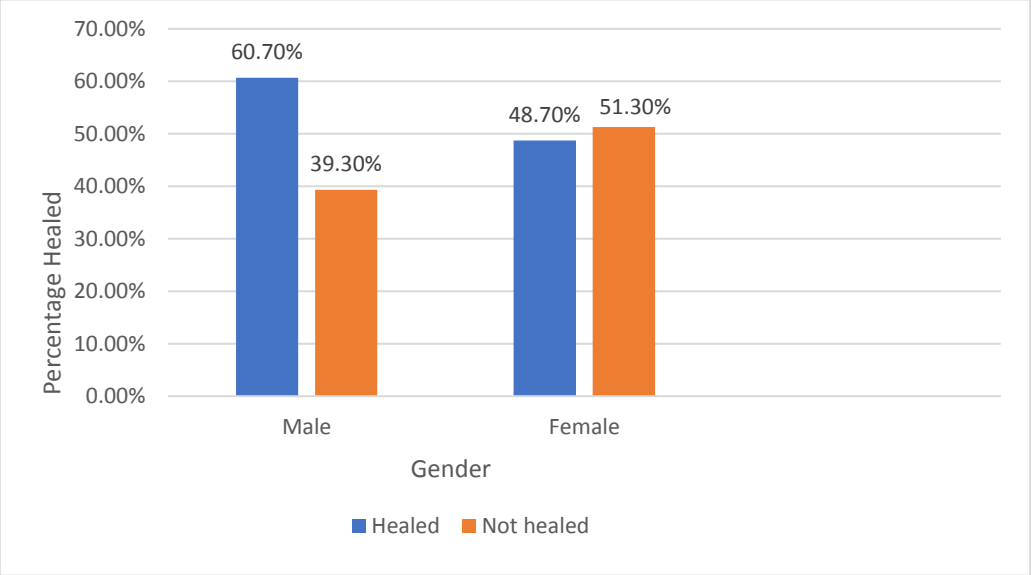


Figure 36 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p value for males and female gender were p=0.159 and p=0.873 respectively.

4.7.3 Association between type of amputation and stump healing in the prospective group

Two types of lower limb amputation were considered - above knee and below knee. The figure 37 shows the association between stump healing and the type of amputation in patients who underwent lower limb amputation. Out of the one hundred patients, fifty three (53%) had an above knee amputation among which healing was seen in 60.4% (n=32). No significant (p=0.216) difference was observed between healed and non-healed stump among the above knee amputation group. The total numbers of below amputations were forty seven (47%) out of which twenty four healed (51.1%) (Figure 37). No

significant (p=0.884) difference was observed between stump healing and below knee amputation. The table below shows distribution of the number of patients who healed their stumps based on the type of amputation. Stump healing was not found to be associated (p=0.349) with type of amputation (Table 34).

Table 34: Type of amputation and stump healing in the prospective group

Type of Amputation	Healed	Not Healed	Total	Chi-Square	*P* value
AKA	32	21	53		
BKA	24	23	47	0.877	0.349
Total	56	44	100		

Table 34 shows the distribution and association between stump healing (healed or not healed) and type of amputation (above knee or below knee). p= 0.349 indicating that stump healing was dependent on the type of amputation.

Figure 37: Stump healing and types of amputation in the prospective group

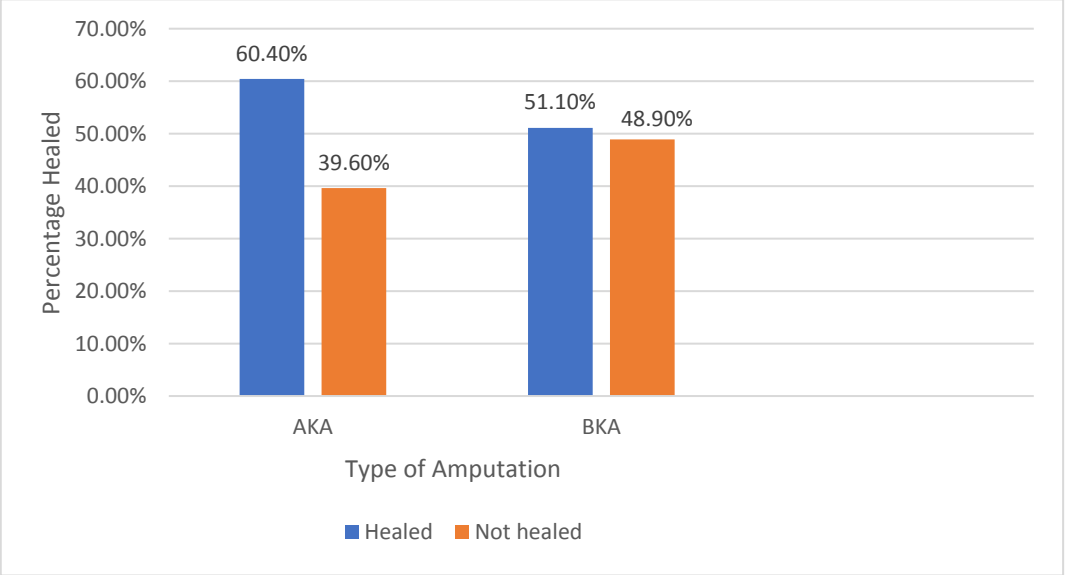


Figure 37 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers

written on top of the bar indicate the percentage. p value for above and below knee amputation were p=0.216 and p=0.884 respectively.

4.7.4 Association between age and stump healing in the prospective group

The figure 38 shows the association between stump healing and gender in patients who underwent lower limb amputation. Of the 100 patients included in the study, 97 were above the age of 50 years (97%) and 3 below the age of 50 years (3%). The youngest patient was aged 43 years and the oldest, 98 years with the average age being 72.28 years. Out of 97 patients above the age of 50 years 53 healed their stump (54.6%) and 44 patient's stumps failed to heal (45.4%) (Figure 38). No significant difference was observed between stump healing and above the age of 50 years (p=0.477). A p value could not be calculated for the group of patients below the age of 50 years. The table below shows an age wise distribution of the number of patients who healed their stumps. The healing of the stump was independent of the age (p=0.119) (Table 35).

Table 35: Age and stump healing in the prospective group

Age	**Healed**	**Not Healed**	**Total**	**Chi-Square**	***P* value**
≤ 50	3	0	3		
> 50	53	44	97	1.130	0.119
Total	56	44	100		

Table 35 shows the distribution and association between stump healing (healed or not healed) and type of amputation (above knee or below knee). p=0.119 indicating that stump healing was independent of age.

Figure 38: Age and stump healing in the prospective group

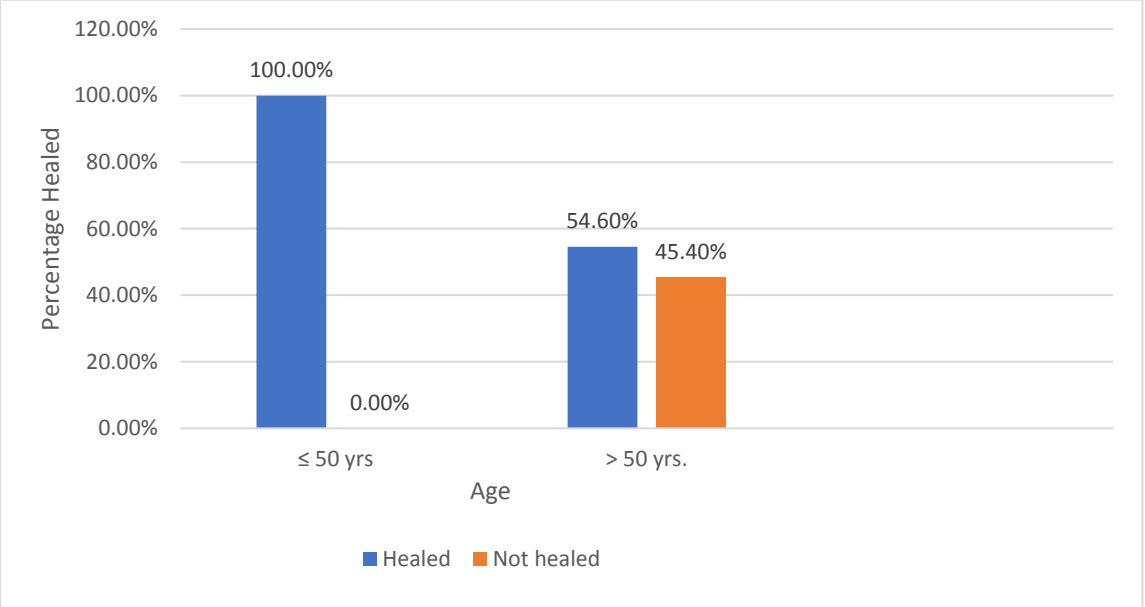


Figure 38 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p value for patients with above the age of 50 years was *p*=0.477.

4.7.5 Association between diabetes mellitus and stump healing in the prospective group

The figure 39 shows the association between stump healing and diabetes mellitus in patients who underwent lower limb amputation. Out of the 100 included patients, there were 44 in the diabetes mellitus cohort and 56 in the non-diabetes mellitus cohort. Among the diabetic population 61.4 % (n=27) healed their stump and 38.6% (n=17) failed to heal their stump. In the non-diabetics 29 healed their stump (51.8%) in comparison to 27 (48.2%) who failed to heal their stump (Figure 39). No significant difference was observed between stump healing and both diabetic (*p*=0.132) and non-diabetic patients (*p*=1.00). The table below shows the distribution of the number of patients who healed their stumps

in the diabetic and the non-diabetic group. Stump healing was independent of Diabetes (p=0.338) (Table 36).

Table 36: Diabetes mellitus and stump healing in the prospective group

Diabetes mellitus	Healed	Not Healed	Total	Chi-Square	*P* value
No	29	27	56		
Yes	27	17	44	0.917	0.338
Total	56	44	100		

Figure 39: Stump healing in patients with diabetes mellitus in the prospective group

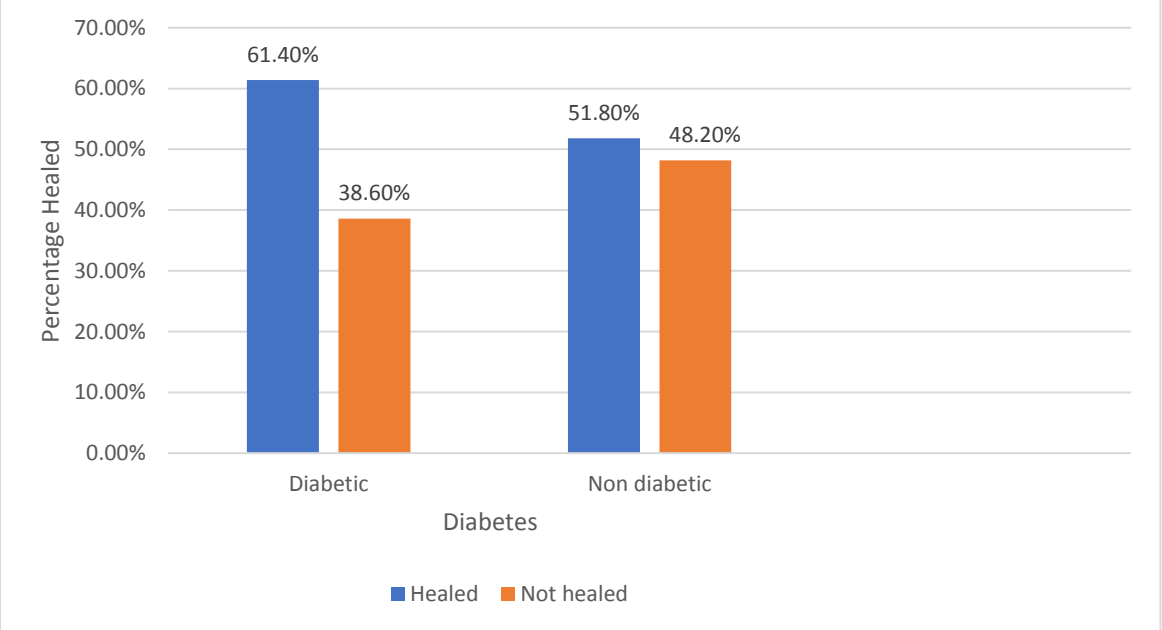


Figure 39 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. The p value for patients with Diabetes and without Diabetes were (p=0.132) and (p=1.00) respectively.

4.7.6 Association between hypertension and stump healing in the prospective group

The figure 40 shows the association between stump healing and hypertension in patients who underwent lower limb amputation. A total of 98 patients were hypertensive and 2 were non hypertensive among 100 patients who underwent major lower limb amputation surgery. In the hypertensive group, the stump healing rate was 56.1% (n=55) while the failure rate was 43.9% (n=43). On the other hand, the healing rate was 50% for both hypertensive and the non-hypertensive group. No significant difference was observed between stump healing and patients with (p=1.00) and without hypertension (p=0.312). The table below shows a distribution of the number of patients who healed their stumps in the hypertensive and the non-hypertensive group. Stump healing was independent of the blood pressure (p=0.863) (Table 37)

Table 37: Hypertension and stump healing in the prospective group

HTN	Healed	Not Healed	Total	Chi-Square	*P* value
No	1	1	2		
Yes	55	43	98	0.030	0.863
Total	56	44	100		

Table 37 shows the distribution and association between stump healing (healed or not healed) and type of amputation (above knee or below knee). p=0.863 indicating that stump healing was independent of hypertension.

Figure 40: Stump healing and hypertension in the prospective group

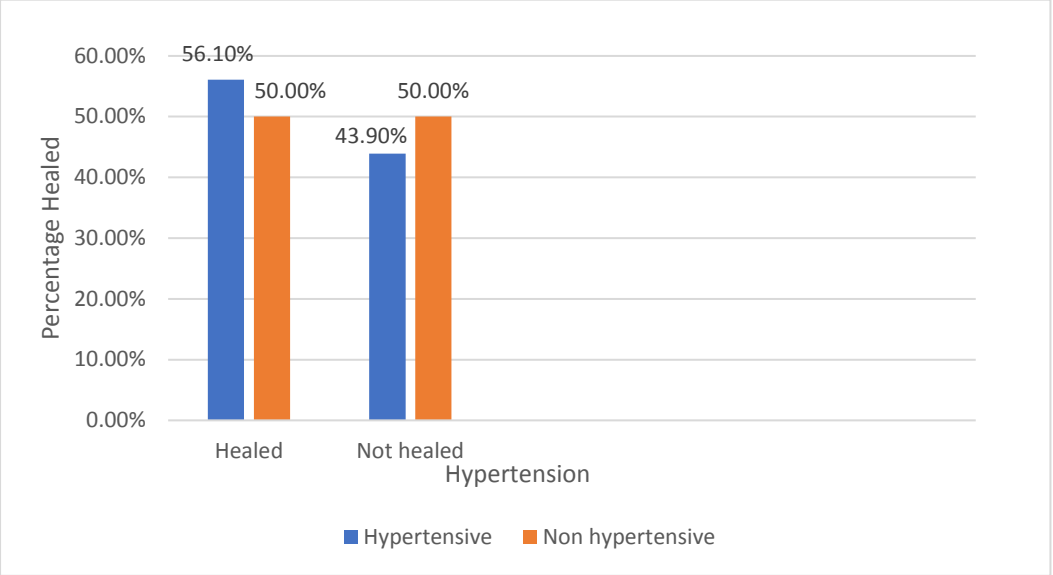


Figure 40 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p value for patients with and without hypertension was (*p*=1.00) and (*p*=0.312) respectively.

4.7.7 Association between smoking and stump healing in the prospective group

The figure 41 shows the association between stump healing and smoking in patients who underwent lower limb amputation. Among the 100 patients, 85 were smokers and 15 were non-smokers. Among the smoking cohort, 52.9% (n=45) achieved stump healing and 47.1% (n=40) failed to heal their stump. The success rate of stump healing was 73.3% (n=11) and 26.7% (n=4) failed to heal their stump among nonsmokers (Figure 41). No significant difference was observed between stump healing and both smokers (*p*=0.588) and non-smokers (*p*=0.197). The table below shows the distribution of the number of patients who healed their stumps among the smoking and non-smoking groups. Stump healing was independent of the smoking (*p*=0.142) (Table 38).

Table 38: Smoking and stump healing in the prospective group

Smoking	Healed	Not Healed	Total	Chi-Square	*P* value
No	11	4	15		
Yes	45	40	85	2.152	0.142
Total	56	44	100		

Figure 41: Stump healing and smoking in the prospective group

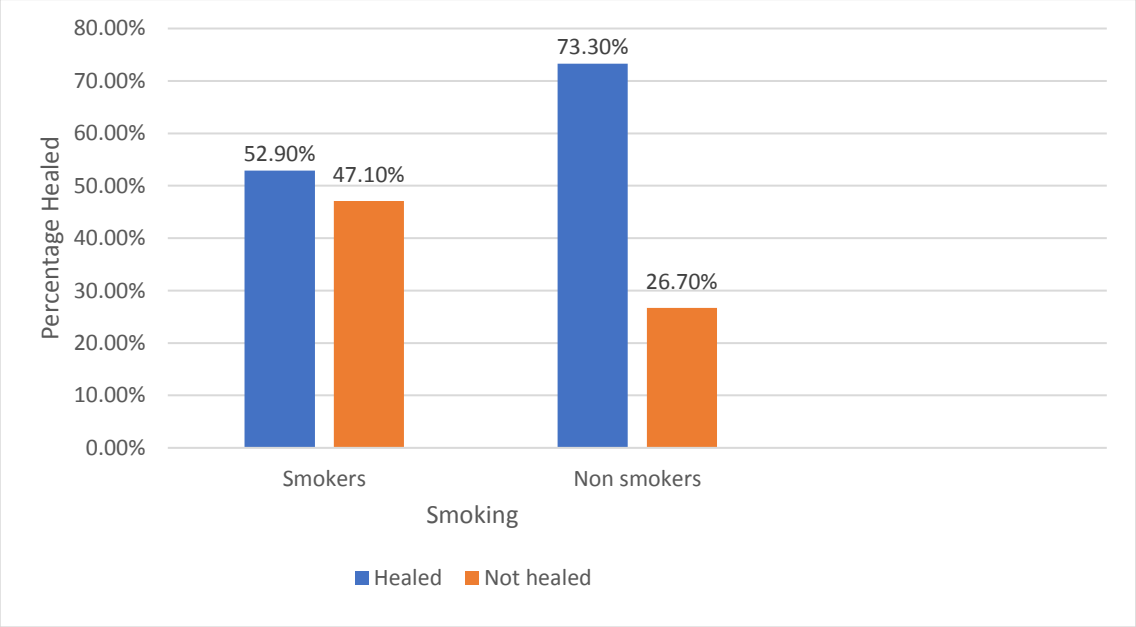


Figure 41 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p value for both smokers and non-smokers was (p=0.588) and (p=0.197) respectively.

4.7.8 Association between HbA1c and stump healing

The figure 42 shows the association between stump healing and HbA1c in patients who underwent lower limb amputation. The impact of the diabetic control was analyzed for the

diabetic patients (n=44) enrolled in the study. HbA1c was divided into three categories namely HbA1c of 6.5% and below, HbA1c between 6.5-7.5% and HbA1c above 7.5%. These groups were based on the patient's diabetic control (optimal/satisfactory and poor) (NICE guidelines. 2015). One hundred percent (n=2) achieved stump healing among the patients with optimal control. The success rate of stump healing was 77.8% (n=12) and 45.8% (n=39) among the satisfactory and poor control group respectively. No significant difference was observed between stump healing and all the three groups; (p=0.301) for the optimal group, (p=0.091) for the satisfactory group and (p=0.722) for the poor control group. The table below shows the distribution of the number of patients who healed their stumps based on their HbA1c. Stump healing was independent of diabetic control. (p=0.057). (Table 39).

Table 39: HbA1c and stump healing in the prospective group

Smoking	**Healed**	**Not Healed**	**Total**	**Chi-Square**	***P* value**
≤6.5	2	0	2		
6.5-7.5	14	4	18	5.746	0.057
≥7.5	11	13	24		
Total	27	17	44		

Table 39 shows the distribution and association between stump healing (healed or not healed) and type of amputation (above knee or below knee). p=0.057 indicating that stump healing was independent of diabetic control.

Figure 42: Stump healing in different HbA1c groups in the prospective group

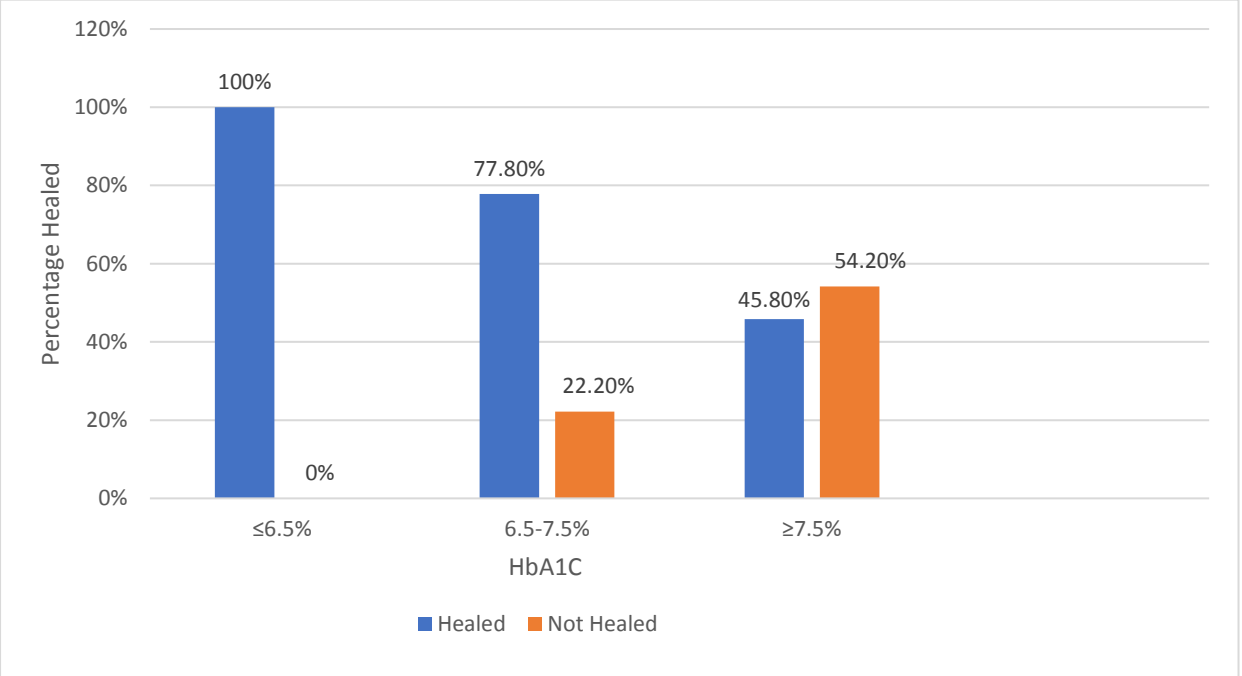


Figure 42 showing association between success and failure of stump healing among subjects who underwent lower limb amputation (above knee and below knee). Numbers written on top of the bar indicate the percentage. p value for the different groups were; p=0.301 for the optimal group, p=0.091 for the satisfactory group and p=0.722 for the poor control group.

4.8 External Validation

Optimism is a well-known problem of predictive models. Their performance in new patients is often worse than expected based on performance estimated from the development data set (Van Houwelingen and Le Cessie. 1990; Harrell *et al.* 1996). Hence the need for external validation. External validation was performed on data obtained from an independent data set of patients who underwent major lower limb amputation surgery at the Royal Infirmary of Edinburgh between year 2010 and 2011. The discriminative ability of the final model for the stump healing was calculated by measuring the area under the Receiver Operating Characteristic curve which is the primary indicator of the model

performance (Bleeker *et al.* 2003). The Receiver Operating Characteristic curve was constructed by calculating the sensitivity and specificity for consecutive cut-off points according to the predicted probabilities from the logistic regression models.

To assess the fit of a logistic regression model is to see what proportion of true positives it classifies as being positive (the sensitivity) and what proportion of true negatives it classifies as being negative (the specificity). Discrimination indicates how well the model discriminates between people with and without the outcome. An Area Under the Curve of 0.5 indicates that the model is not discriminating very well (no different to tossing a coin); an Area Under the Curve of 1.0 indicates perfect discrimination. Receiver Operating Characteristic curve for external validation was developed, based on the predicted probabilities for every patient and calculating the sensitivity and specificity for consecutive cut-off points according to the predicted probabilities from the logistic regression models.

$$Predicted\ Probability = \frac{e^{(\beta_0+\beta_1X_1+\beta_2X_2+\cdots+\beta_nX_n)}}{1+e^{(\beta_0+\beta_1X_1+\beta_2X_2+\cdots+\beta_nX_n)}}$$

$$Predicted\ Probability = \frac{1}{1+e^{-(\beta_0+\beta_1X_1+\beta_2X_2+\cdots+\beta_nX_n)}}$$

Where

B_0 is the intercept, β_1 till β_n are the regression coefficients and X_1 to X_n are independent variables.

$$Predicted\ Probability = \frac{1}{1+e^{-(-1.82+(0.563XSe\ Na)+(0.509X\ Se\ Creatinine)+(0.561\times HDL)}}$$

Se Na=serum sodium levels, Se Creatinine=serum creatinine levels and HDL=serum High Density Lipid cholesterol.

By using the above formula, predicted probability of each patient was calculated. The area under the Receiver Operating Characteristic curve for the prediction of stump healing was calculated using predicted probabilities and patients' outcomes (healed or not healed) of each patient.

Figure 43: Receiver Operating Curve analysis for validation of the prognostic model

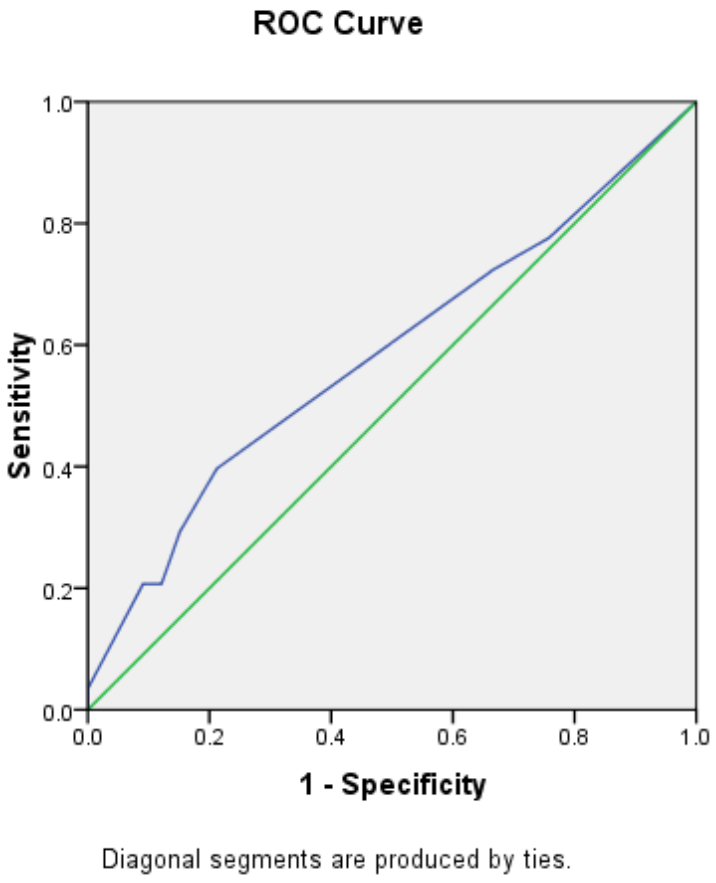


Figure 43 showing receiver operating characteristics of stump healing. Area under the curve was 0.584 (95% CI: 0.365-0.612), indicating good discriminatory ability of stump healing.

The area under the Receiver Operating Characteristic curve for the prediction of stump healing in lower limb amputation in the validation set was 0.584 (95% bias-corrected CI: 0.365-0.612), consistent with good model discrimination (Figure 43).

CHAPTER 5

DISCUSSION

The results of this study identified 3 blood markers namely serum sodium, serum creatinine and serum High Density Lipid cholesterol to be useful predictors of lower limb stump healing out of 7 markers which were noted to have association with stump healing in multivariate analysis. Each of these markers were independently associated with stump healing following a major lower limb amputation. Healing of the stump as defined was achieved in sixty three percent (n=189) patients. The healing rates were noted to be 68.3% (n=109) in the above knee amputation group and 56.8% (n=80) in the below knee amputation group.

All the biomarkers markers used were interpreted as dichotomous variables rather than as a continuous variable due to the methodology of the study. According to Harrell *et al.* (1996) for continuous predictors (independent variables), logistic regression assumes that predictors are linearly related to the log odds of the outcome. However, if this assumption is violated, logistic regression underestimates the strength of the association and rejects the association too easily, that is being not significant where it should be significant. Hence it is preferable to categorize the continues variables into dichotomous data. The authors of this study however, realize that in conversion of the continuous variables into categorical data, useful information could be lost.

5.1 Gender and stump healing

In the present study, gender emerged as a significant marker for the prediction of stump healing in univariate analysis but not in multivariate analysis. In other words, gender had a strong association with stump healing on its own but when the relative contribution of each of the predictors to the total variance was seen, it showed no association. This could be the effect of unbalanced sample size with the males being in higher proportions

compared to the females (n=214 versus n=86). Wang *et al.* (1994) who carried out a prospective study evaluating parameters capable of predicting wound healing in patients with peripheral vascular disease in forty-four amputations also noted that gender was not associated with predicting wound healing. In addition, Eneroth (1993) in a study on 177 patients with amputated limbs looked into factors which could potentially reduce the average age for amputation surgery and which could indicate success rate of lower limb amputations in patients with vascular disease also reported a similar finding. The lack of association in the above studies could be due to a small sample size and a larger prospective study could have different outcomes.

There are other studies that showed that gender was a significant marker for lower limb stump healing. According to López-de-Andrés *et al.* (2011), who looked at 90,064 non-traumatic amputations between 2001 and 2008, (46,536 minor and 43,528 major) performed in Spanish population reported that men were more likely to experience complications of lower limb amputations, including poor stump healing than women (male-to-female ratio being >2:1 (Type 1 diabetes mellitus: 2.23, Type 2 diabetes mellitus: 2.18). Vamos *et al.* (2010) studied the incidence of lower extremity amputations in individuals with and without diabetes mellitus in England between 2004 and 2008. They observed that the incidence of lower limb amputations was significantly higher among men than among women with diabetes mellitus ($p<0.001$). A study by Faglia *et al.* (2001) who reviewed a total of 115 subjects suffering from diabetes mellitus from 1990 to 1993 with a new major amputation and their survival rates noted an association with stump healing and female sex ($p=0.027$) in the multivariate analysis. A study undertaken in the Netherlands by van-Houtem *et al.* (2004) indicated a gender trend in amputees. Reviewing data for 1991–2000, the researchers reviewed a total of 2,409 amputations. In 1991, there

were 818 male amputees and 869 female amputees; in 2000, the number of male amputees had risen to 971 but for women it had fallen to 702. The differences are statistically significant ($p<0.001$). One possible explanation for the increase in male amputations is diabetes mellitus had increased proportionately more in men between 1991 and 2000 (114,000 to 211,000 cases, representing an increase of 84.9%) than in women (193,000 to 251,000 cases; 29.9% increase).

The growing prevalence of lower extremity amputation among men, as observed in this study and other studies quoted above however, cannot be attributed solely to the increasing incidence of diabetes mellitus in men. Delayed manifestation of atherosclerotic disease in women assumed secondary to the exposure to endogenous oestrogens during the fertile period of life could also be contributory. The research findings of Margolis *et al.* (2002) suggested that, in comparison to postmenopausal women who did not take hormone replacement therapy, those that did had a lesser risk of developing venous and pressure ulcers. Furthermore, in patients who took hormone replacement therapy, the age-based relative risk prediction for formation of venous and pressure ulcers was 0.65 (95% CI, 0.61 to 0.69) and 0.68 (95% CI, 0.62 to 0.76), respectively. Several other studies including Pirila *et al.* (2002) noted beneficial effects of oestrogen on wound healing. Mechanisms underlying the effects of oestrogen on the complex process of wound healing have not been fully established but the anti-inflammatory properties including suppressive effect of oestrogen on Poly Morphonuclear Leukocytes (PMN) chemotaxis play a role (May *et al.* 2006).

5.2 Type of amputation and stump healing

In this study, sixty three percent (n=189) of lower limb stumps healed. The trans-femoral

amputation (above knee amputation) healing rate was better than the trans-tibial amputation (below knee amputation) (68.3% vs. 56.8%). This study's findings that primary healing rates are better in above knee stumps in comparison to below knee stumps are consistent with other studies including that of Dormandy *et al.* (1999) who added that a wide variation was exhibited by the rates of healing of amputations conducted below or above knee. Above-knee amputations have been shown to heal in 90% of cases, whilst the healing rate of below-knee amputations varies greatly, from 30% to 92%, an average being 60% in below knee amputations. VanRoss *et al.* (2009) who conducted clinical trials in 250 new lower-limb amputees reported that the healing rates in below knee stumps were 74%. These results appear to be logical form an anatomical perspective wherein the higher the amputation the better the blood supply.

However, there are other studies where stump healing was noted to be better in below knee amputation. According to Jensen and Mandrup-Poulsen (1983) the success rate of below knee amputations with regards to stump healing and prosthetic fitting was 83% in contrast to 69% healing rates and prosthesis fitting in above knee amputation. Similar findings were reported in other studies (Christensse, 1976). Burgess *et al.* (1971) who reviewed 177 consecutive patients who underwent lower limb amputations noted that out of 145 below knee amputations, only 12 failed to heal compared to 8 of the 40 that had above knee amputation. Chilversa *et al.* (1971) reviewed 53 lower limb amputations and postulated that a below knee amputation was the most feasible option in the case of patients suffering from ischaemia of the lower extremities, because it had a healing rate of 70%. Though the healing rates for above knee stumps were better than the below knee in this study, the level of amputation was found to be a significant marker for stump healing only in the univariate analysis.

The generalisation that above knee amputation generally have better healing rates and lower reoperation rates compared to below knee amputation stems from the fact that the vascular supply decreases distally in patients with peripheral arterial disease. However, when tissue ischaemia or infection proximal to the below knee amputation level is evident, above knee amputation is also the level of choice for lower limb amputation (Rosen *et al.* 2014). However, several other studies reported that above-knee amputation was not only correlated with a higher mortality rate, potentially as a result of advanced and severe disease, but was also more prevalent among elderly patients. One such study was that undertaken by Pell *et al.* (1999), which investigated 2759 patients from Scotland who had major amputation during the period 1989-1993 due to peripheral arterial disease supported this. The main causes of death associated with above- and below-knee amputation were identified by Rush (1981) as being myocardial infarction and sepsis, respectively. Meanwhile, Dormandy (1991) suggested that the risk factors most likely associated in death within three months after major amputation were ischaemic heart disease and diabetes mellitus. On the other hand, the fact that cardiovascular disease, cerebrovascular disease and diabetes mellitus become more prevalent with age provides an alternative explanation for the higher mortality rate among older patients that has been reported by numerous studies.

In this study, the healing rates were better in the above knee amputation group. As this study did not look into arterial imaging of the lower limb, it was presumed that the patients with above knee amputation had good blood supply along the femoral arteries. There could also be variation secondary to the previous vascular procedures which the patients would have undergone previous to the amputation surgery. Most of the above-mentioned studies also did not exclude patients with previous vascular procedures as this would dwindle

sample size. There are however other factors that prompts the surgeon to perform a above knee amputation despite having good distal circulation. These include poor possibility of harnessing skin flaps around the site of the amputation. The reason behind the decision for the level of the surgery was not taken into account in this study. Future studies could look into the indications for the level of surgery and correlate the stump healing with the indication.

One of the key parameters for the level of amputation being the blood supply has not been looked at in this study. As a result, the outcomes of this study only aid the surgeon to decide the level of amputation but does not provide conclusive evidence for an ideal level of amputation.

5.3 Age and stump healing

The age of the patients who underwent lower limb amputation surgery varied between 34 and 97 years, the mean age being 70.16 ± 14.5 years. In this study, age was not found to be a predictive marker for stump healing both in univariate and multivariate analysis. Similar findings were reported by Eneroth *et al.* (1993) who prospectively analysed stump healing in 177 cases and noted that age had no relation with healing rates. Low *et al.* (1996) who reviewed 60 below knee amputations in patients with diabetes mellitus also reported that age did not play a role in stump healing (p=0.40). However, this was an observational study with a small sample size.

However, other studies including a study by Taylor *et al.* (2005) looking into preclinical factors predicting functional outcomes in 553 patients who had lower limb amputation and they concluded that older patients aged more than 70 years who experience non-ambulatory status were 10 times less likely to heal their stump or wear prosthesis. Chen

et al. (2008) did a five year review (2002-2006) on factors effecting stump healing and functional outcomes post lower limb amputation. One hundred seventy-nine patients were included whose ages ranged from 28 to 85 years (average 64.3 ± 12.9 years) and the mean follow-up time was 28.3 ± 13.0 months. They added that unchangeable factors including age significantly affected stump healing ($p<0.001$).

In a retrospective study of all non-traumatic amputations in patients with (n=100) and without (n=151) diabetes mellitus performed between 1990-1995, Tentolouris *et al.* (2004), noted that patients without diabetes mellitus who had lower limb amputation were older than patients who suffered from diabetes mellitus who underwent the same procedure. They added that the older patients also went on to have higher revision rates secondary to poor stump healing ($p=0.001$). In other words, amputations in individuals with diabetes mellitus are performed at a younger age, a finding observed in other studies including a retrospective study by Mayfield *et al.* (2001) who looked at the common demographic and comorbid conditions that affect survival following non-traumatic amputation. They added that survival following a lower-limb amputation is impaired by advancing age secondary to poor wound healing and other co-morbidities including cardiovascular and renal disease. Faglia *et al.* (2001) reviewed a total of 115 subjects with diabetes mellitus from 1990 to 1993 for new ulceration and new major amputation and their survival rates and noted their association with ankle-brachial index ≤0.5 ($p=0.005$), age ($p=0.003$), and female sex ($p=0.027$) in the multivariate analysis. Pell *et al.* (1999) studied 2759 patients undergoing major amputation between 1989 and 1993 for peripheral arterial disease. The study reported that sixty percent of amputations (n=924) performed in patients under 65 years of age were below knee amputations, compared to 53% (n=621) in those over the age of 80 years undergoing above knee amputations. Thus, proximal

amputation was found to be more common in older patients. They concluded that age was an independent predictor of death at 30 days ($p<0.0001$), 6 months ($p<0.001$), 12 months ($p<0.0001$) and 2 years ($p<0.0001$) post-operation secondary to poor stump healing and other co-morbidities. Patients in the above knee amputation group were older than those in the below knee amputation group (mean age of 77 years compared to 69 years, $p=0.039$). Though this was large sample study, it had a retrospective study design.

Duration of diabetes mellitus and advancing age independently predicted diabetes mellitus morbidity and mortality rates. According to Elbert *et al.* (2014) for a given age group, the rates of each complication including lower limb amputation increased dramatically with longer duration of the disease (1.28 per 1000 person-years vs 4.26 per 1000 person-years). According to Fowler (2008) with advancing age and worsening diabetic neuropathy and atherosclerosis, the incidence of macrovascular complications including lower limb amputation increased accordingly.

The finding that advancing age impaired stump healing could be explained by the fact that ageing generally impairs wound healing. The manner in which body systems, environmental stresses and disease interact with the ongoing process of aging increases the likelihood of older patients experiencing difficulties with wound healing. Several studies have provided evidence that diminishing levels of glycosaminoglycan and collagen, changes in the physical attributes of collagen and elastic fibres, and disruption in the organisation of the microcirculation all contribute to delayed healing in older individuals (Minimas. 2007).

However according to Hasanadka *et al.* (2011), who carried out a study in 4250 (2309 below knee amputations and 1941 above knee amputations) patients over a 3 year period

younger patients (age group 50-59) were almost two times (Odds Ratio-1.9) more prone to having a wound occurrence after below knee amputation compared with their older age group comparators. Explanations for increasing wound occurrences according to them in this younger age group included having more aggressive atherosclerotic disease, subsequent early failure of revascularizations, and the combination of genetic predisposition, hypercoagulability, and virulent risk factors. They added that inappropriate delay in amputation from an overly aggressive desire to save the foot in a younger patient, difficulties in care compliance in a more physically active age group, biased belief that this group of patients may not need the same skilled longer-term care and rehabilitation to heal a below knee amputation compared with older counterparts could also be the reasons for their findings.

This study had a cut of age of 50 years and patients were categorised accordingly into age below and above 50 years. This is in keeping with several other studies conducted in diabetic foot ulcer patients. It would be interesting to note if the same trend of healing would have been found if the age cut of was to be changed to say 65 years. It would also mean that the group numbers would also be relatively evenly matched thereby making the results better interpretable. This is one of the limitation of this study. Also, as mentioned, age here was used as dichotomous variable rather than a continuous variable due to the methodology used in this study. The authors agree that in doing so useful information could have been lost.

5.4 Diabetes mellitus and stump healing

Diabetes mellitus remains a risk factor for lower limb amputation. In this study, 138 subjects had diabetes mellitus and 162 did not have the disease. The success of stump

healing in these groups was 61.6 % (n=85) and 64.2 % (n=104) respectively.

Many studies have concluded that diabetes mellitus is an independent risk factor for foot ulcers and lower limb amputation. However, few studies have been done with regards to diabetes mellitus as a risk factor for stump healing. According to Low *et al.* (1996) who reviewed 60 below knee amputations in patients with diabetes mellitus, many risk factors for foot ulcer healing were common to those of stump healing in lower limb amputation. In other words, though there was an anatomical difference in the sites, the physiological factors that played a role in healing of a foot ulcer and a stump were the same. Reiber *et al.* (1992) who reviewed eighty patients with lower limb amputation associated with diabetes mellitus over a 30 month period in a case control study reported that diabetes mellitus was a predictor of stump healing (p=0.01), a finding shared by other studies including Moss *et al.* (1992) who investigated risk factors for lower extremity amputations in a cohort study (95% CI, 1.0-3.2), though both these studies were limited by their study design. Criado *et al.* (1992) who reviewed 79 patients with diabetes mellitus who underwent emergency lower limb amputation procedure for severe infection of the foot noted that diabetes mellitus was a risk factor for poor wound healing. However, this study had a small sample size (n=79). Similar findings were also reported by Apelqvist *et al.* (1992) who carried out a prospective study on 314 sequentially presenting patients with diabetic foot ulcers.

In a study by Tentolouris *et al.* (2004), of the 257 amputations performed during 1990–1995, 39.7% (n=102) were in patients with diabetes mellitus. They reported that more patients without diabetes mellitus had major amputations in comparison with patients who suffered from diabetes mellitus (62.3% vs 48.7%). However, often patients with diabetes mellitus had two or more amputations during the study period in comparison with non-

diabetic patients (54.9 vs. 36.4%, respectively) due to failure of the stump healing. They also reported that the duration of diabetes mellitus was an independent predictor of mortality in the diabetic group (p=0.05). However, this was a retrospective and observational study, potentially suffers from the limitations of such observations. Cause-specific mortality was also not examined in this study.

In a study by Heikkinen *et al.* (2007), the mortality rate following major lower limb amputation was higher among vascular patients with diabetes mellitus than among non-diabetics of both sexes, especially among male patients, despite the fact that those without diabetes mellitus were older than those with diabetes mellitus (mean age 76.7 and 73.2 years, respectively, $p<0.01$). In other studies, coronary heart disease and stroke were the major reasons for the elevated mortality rate associated with diabetes mellitus (Mulnier *et al.* 2006). High HbA1c which shows poor diabetes mellitus control was strongly associated with atherosclerosis and was also strongly related to Low Density Lipid cholesterol and other cardiovascular risk factors. Several studies have also shown that improvements in glycaemic control can slow progression of atherosclerotic disease in individuals with both Type 1 (Larsen *et al.* 2004) and Type 2 diabetes mellitus (Wagenknecht *et al.* 2003).

Although the above studies suggested a link between diabetes mellitus and stump healing, other studies found no relation between them. Eneroth *et al.* (1993), prospectively analysed stump healing in 177 cases who underwent lower limb amputation and observed that diabetes mellitus had no relation with healing rate. Of their sample size, 40% (n=70) of patients had diabetes mellitus. The relative risk reduction they noted for diabetes mellitus was 0.5 (95% CI of 0.1-0.8) which was lower than all the other factors which they looked into (sex, level of amputation, smoking, preoperative blood pressure, serum

creatinine, erythrocyte sedimentation rate and blood glucose). However, they did not exclude patients who had a re-amputation at the same or higher level which could potentially have skewed the results. They, however added that despite having no association with stump healing, diabetes mellitus lowered the mean amputation age by 3.2 years (p=0.041). Wang *et al.* (1994) performed a prospective study on 44 amputations performed on 38 patients evaluating parameters predicting wound healing in patients with peripheral vascular disease. They reported no association between duration of diabetes mellitus and stump healing (p=0.021). However, these findings were collated based on a sample size of only 20 patients with diabetes mellitus. Low *et al.* (1996) reviewed 56 patients with diabetes mellitus who underwent below knee amputations. They noted that duration (≤ 10 year versus ≥10 years) of diabetes mellitus (p= 0.27) and type of diabetes mellitus (Insulin dependent versus independent) (p=0.44) was found to have no predictive value on below knee amputation healing rates. This study however was retrospective in nature and the sample size was small.

In this study, diabetes mellitus was not found to be a significant predictor of stump healing both in univariate and multivariable analysis. It could be argued that the foot and the lower limb stump are two different anatomical sites and that healing in these two sites could well be affected by a different set of factors. The blood supply for example is better in major blood vessels proximally and would decrease peripherally specially in diabetic patients who are known to have diffused atherosclerosis. Physical factors like offloading which are more relevant in the case of diabetic foot ulcer healing would also play a role in healing. This study did not take into account the duration and the type of diabetes in patients which could well play a role in healing. This is one of the limitation of this study.

5.5 Hypertension and stump healing

In this study, essential hypertension was noted in 283 patients. In their analysis of a sample size of 110 patients suffering from peripheral vascular disease who underwent a lower limb amputation in the period 1987-1990, Lee *et al.* (1992) concluded that hypertension was more likely to have determined amputation than diabetes mellitus, as it was found to be more frequent than the latter (32 cases of hypertension, as opposed to 10 cases of diabetes mellitus). The considerable prevalence of hypertension led the researchers to suggest that future treatments of peripheral vascular disease should put more emphasis on effective control of hypertension, as a method of preventing amputation. Frugoli *et al.* (2000) who looked into the cardiovascular risk factors in 170 amputees noted that hypertension was higher in individuals with amputated limbs, with 42.7% (n=71) in contrast to 23% (n=39) in those without amputations. According to Tseng *et al.* (1994) who reviewed Chinese patients with diabetes mellitus after lower extremity amputations from 1982 to 1991, a history of uncontrolled hypertension was found to be an indicator for predicting a fatal outcome in relation to poor stump healing and mortality after a lower limb amputation with rate ratios of over two-fold. However, in this study hypertension was found to be an independent risk factors for stump healing in univariate analysis but not in multivariate analysis.

Hypertension, however is a well-known risk factor for the development of atheromatous peripheral arterial disease. In patients presenting with peripheral arterial disease, hypertension is a major associated cardiovascular risk factor, present in up to 55% patients with peripheral arterial disease who are at risk of blood pressure-attributable progression of the peripheral vascular problems including high risk of death and disabling ischaemic events. According to Frugoli *et al.* (2000) three risk factors were elevated above the United

States population norms among the amputee population, namely; cholesterol, hypertension, and diabetes mellitus. About 47% of amputees suffered from hypertension which contributed to their higher cardiovascular mortality. According to Meijer *et al.* (2000) who looked into the atherosclerotic risk factors which were determinants for peripheral arterial disease in 6450 subjects noted that systolic blood pressure after multivariate analysis was a major risk factor and conferred an odds ratio for PAD of 1.3(95% CI 1.2–1.5) per 10 mmHg systolic pressure. Many studies report that blood pressure management in peripheral arterial disease tends to be poor irrespective of the presence of diabetes mellitus as a comorbidity (Ostchega *et al.* 2004). In the PARTNERS study by Hirsch *et al.* (2001), hypertension was less often treated in new (84%) (n=312) and prior peripheral arterial disease (88%) (n=264) patients compared to treatment of hypertension in subjects with cardiovascular disease (95%; $p<0.001$). This may result in an increased incidence of lower limb amputation in patients with peripheral arterial disease and diabetes mellitus.

The groups with hypertension and without hypertension were not evenly matched in this study. Hence the result will need to be interpreted with caution. The control of hypertension including anti-hypertensive medications was also not looked into, though most of the patients had good control (BP of less than 150/90).

5.6 Smoking and stump healing

Hughson *et al.* (1978) argued that smoking was a major risk factor for intermittent claudication. The association between smoking and healing of lower extremity amputation was investigated by Lind *et al.* (1991) based on a review of 165 primary above- and below-knee amputations among a number of 137 patient. Results showed that, by comparison to

the patients who did not smoke, those who did were 2.5 times more likely to undergo a further amputation.

Stewart (1987) confirmed that the incidence of amputation not only higher among patients who smoke, but was also required at a younger age. In a seminal paper looking at the effect of smoking in patients with diabetes mellitus with lower limb amputation, Liedberg and Persson (1983) looked at 188 lower limb amputees in Lund, Sweden and concluded that smoking of cigarettes positively correlated with a higher incidence of intermittent claudication ($p<0.001$) and that heavy smokers (defined as 10 cigarettes or more a day or 10 g tobacco a day) were at three times the risk of developing intermittent claudication compared with non-smokers.

Smokers with peripheral arterial disease exhibited a lower physical performance during treadmill assessment, including maximum oxygen uptake and quicker onset of claudication pain as a result of walking (Shimada *et al.* 2011). Meanwhile, Lu *et al.* (2013) reported that, by contrast to non-diabetics, patients with diabetes mellitus were considerably more likely to develop peripheral arterial disease, cigarette smoking enhancing this likelihood by an additional 50%. Identification of the determinants of the success rate of lower extremity amputation in individuals with vascular disease was the focus of the study conducted by Eneroth *et al.* (1993). To this end, the authors recruited 177 number of patients who had undergone amputation; half of these never smoked, while 26% and 24% were active and past smokers, respectively. Men accounted for 87% of the smokers. The average age at amputation was 59 and 74 years, respectively, in the case of individuals who smoked more than 15 cigarettes per day and those who smoked less. However, despite the fact that the difference between the two groups was non-significant (p=0.08), the failure rate at 6 months following amputation was lower among patients who

had never smoked (18%) than among those who smoked (31%). In addition, the likelihood of failure was 2.1 lower in patients who had never smoked by comparison to smokers (95%CI 0.3-13), and individuals were more likely to undergo amputation at a younger age by 8.6 and 3.2 years, respectively if they smoked (p=0.0001) or had diabetes mellitus (p=0.041).

Wound healing is delayed by smoking (Sørensen, 2012), current smokers being more likely to experience incomplete healing of amputation stump (VanRoss *et al.* 2009; Hasanadka *et al.* 2011). Observations like these regarding the implications of smoking serve to highlight the importance of ongoing smoking cessation campaigns, even after healing of the primary amputation despite the fact that documentation of the correlation between smoking and contralateral amputation risk is yet to be established (Lind *et al.* 1991). In a prospective study that investigated stump healing in 177 cases, Eneroth (1999) found that high levels of nicotine in blood reduced the blood flow speed, enhancing the likelihood of microthrombus formation. Furthermore, according to the findings of other studies, in contrast to individuals who did not smoke, those who did had higher levels of carboxyhaemoglobin. Carboxyhaemoglobin has been proposed as a determinant of wound infection and healing probability due to promotion of relative hypoxia which led to wound hypoxia (Sorensen *et al.* 2003). The cigarette components of nicotine, carbon monoxide and tar have an adverse effect on the functions of several endothelial cells including Connective Tissue Growth Factor (CTGF), eventually compromising their anticoagulation and prevention of clot formation capabilities, as well as diminishing their fibrinolytic activity. Additionally, the endothelium-dependent mechanism of vasodilation is also affected by smoking through interference with processing and release of nitric oxide. The compression, multiplication and movement of vascular smooth muscle cells can be

enhanced in conditions with reduced levels of nitric oxide (Newby, 1999).

In this study, smoking was identified as an important indicator of stump healing only in the univariate analysis. According to Lo *et al.* (1995) one of the possible explanations for variables to show a tendency wherein they are significant in univariate analysis but not in multivariate regression analysis or vice versa could be if the sample size is unbalanced. In this study, the number of patients who smoked (n=245) were compared to the non-smoking cohort (n=55) resulting in an unbalanced sample size. The smoking status was obtained from the data software in the retrospective study. This meant that important information like number of pack years, the duration of smoking and the type of smoking was not available. This could have potentially biased the results.

5.7 Kidney function markers (including electrolytes) and stump healing

In this study, serum urea was not shown to be a predictive marker in lower limb stump healing both in univariate as well as multivariate analysis. However, serum creatinine was found to be a predictor for lower limb stump healing both in the univariate as well as the multivariate analysis. The healing rate of patients with a normal concentration of serum creatinine increased by 66% (OR 1.664; 95% CI 0.94 to 2.946). Taylor *et al.* (2005) reviewed the correlation between preoperative clinical parameters and post-surgery results with regards to limb functionality in the case of patients who had been subjected to leg amputation, and concluded that patients suffering from end-stage renal disease performed poorly with transtibial amputation secondary to poor stump healing and recommended palliative transfemoral amputation instead. Renal failure is considered to be an important factor leading to amputation following stump healing complications. Blume *et al.* (2007) stated that end-stage renal disease and the failure of amputation stumps to heal exhibited

a strong correlation (p=0.0209). However, this study was restricted by its study design (retrospective study) and sample size (n=80). Similar findings were also reported in other studies at lower levels of lower limb amputations (Hodge *et al.* 1989). According to Steven (2000) uraemia caused significant impairment of the healing process. The adverse effects of uraemia were as a result of the changes in enzyme systems, biochemical pathways, and cellular metabolism. Different haemostatic disruptions are considered to be the cause of bleeding diathesis and pro-thrombotic condition in uraemia. However, Eneroth *et al.* (1993) in a study on 177 patients with amputated limbs looked into factors which could potentially reduce the average age for amputation surgery reported that kidney function played no role in lower limb stump healing. This could potentially be because of the mild degree of renal impairment in the subjects involved in this study (mean creatinine levels 120).

Serum sodium was found to be an important predictor for stump healing. Patients with a normal concentration of serum sodium had a lower limb stump healing rate of 75% (n=225) in comparison with those with an abnormal concentration of serum sodium (OR 1.756; 95% CI 1.048-2.942; p=0.031). Yaghoubian *et al.* (2007) also made comparable observations in individuals with necrotising soft tissue infection who had had an amputation procedure. diabetes mellitus along with abnormal electrolytes interferes with the normal functioning of the metabolism, promoting pro-thrombotic state, endothelial function impairment, growth factor deregulation, and excessive deposit of extracellular matrix. The deranged serum sodium in patients who underwent major lower limb amputation in this study is probably as a consequence of the disruption to the metabolism secondary to the injury post a major surgery. It could also be a reflection of the systemic illness of the patient given the multiple comorbidities they suffer from. According to

Nissen *et al.* (1992) the preoperative physical condition and the presence of comorbidity influences not just the stump healing but also the functional outcomes following a lower limb amputation surgery including the prosthesis wearing rates. This was also observed in another study done by Chen *et al.* (2008) who did a five year review (2002-2006) on factors effecting stump healing and functional outcomes post lower limb amputation on one hundred seventy-nine patients whose ages ranged from 28 to 85 years (average 64.3 ± 12.9 years). They added that in their series, the renal function factor (creatinine >1.4 mg/dl or worse, p=0.045) affected the pre-prosthetic training waiting time (p=0.001) and also influenced the daily prosthesis usage time (p=0.01). This study however was limited by its study design (retrospective analysis). Other studies including a study done by Sheahan *et al.* (2005) who looked at 670 patients who underwent 920 minor amputations on 747 limbs noted that end-stage renal disease (serum creatinine levels >2.0 mg/dL) was an independent risk predictor (p<0.0001, OR 1.72, 95% CI 1.12-2.83) for limb loss and a further amputation at a higher level due to poor stump healing. Though this study had a large sample size, it was retrospective in nature.

5.8 White cell count and stump healing

In this study, white cell count was not noted to be of prognostic value in stump healing in lower limb amputation both in univariate as well as multivariate analysis. It could be argued that advanced atherosclerosis and poorly controlled diabetes mellitus are immune-compromised states and the patients might not mount a response to infection and this could result in a minimal rise in the inflammatory markers which could explain the finding in this study. According to Calhoun *et al.* (2009), in chronic inflammation the blood leukocyte count is usually normal and most of the diabetic foot ulcers with both soft tissue and bony infections are chronic. Chronic infection is the result of the co-existence of

infected, nonviable tissues and an ineffective host response.

Low *et al.* (1996) who reviewed 54 patients with diabetes mellitus with 60 below knee amputations in 1992 also reported that white cell count was not associated with stump healing (p=0.17). According to Tentolouris *et al.* (2004), however there is an independent association between a higher white cell count and survival in the amputees without diabetes mellitus. They found no association between higher white cell count and mortality in patients with diabetes mellitus.

One of the explanations for studies not finding an association between inflammatory markers and stump healing could be because the degree of variation of infection itself in the studies. According to Ince *et al.* (2008) who compared populations and outcomes of diabetic foot ulcers managed in the United Kingdom and other countries including Germany from a series of 449 patients concluded that degree of infection varies considerably across different studies. There are also discrepancies in management of an infected diabetic foot ulcer. The degree of infection (presence of soft tissue versus bony infection) plays an important role not just in the treatment options but also on the outcome of the infection. Local microbiological susceptibility and epidemiology dictate the types of antibiotics that can be used. However, despite the importance of pathogenic agents and epidemiology in the selection of antibiotics, antibiotic administration cannot wait until culture and sensitivity results are generated. Hence, local epidemiological and susceptibility data are essential for preliminary empirical treatment. According to Lipsky *et al.* (1999) the type of organism grown in the culture also plays a role in the severity of the infection. With so many factors playing a role in the management of infection control, variable outcomes are expected which will have an impact on the inflammatory markers and stump healing.

However, other studies have shown an association between raised inflammatory markers and poor stump healing. Of the 80 patients examined by Blume *et al.* (2007), 10% (n=8) had an abnormal number of leukocytes and slowed down the rate of limb healing, while a proportion of 68% (n=13 of 19) displayed no stump healing. These findings led to the proposition that leucocytosis and lack of amputation stump healing were correlated (p=0.0052). However, this was a retrospective study and the sample size was small. The correlation between nutrition and lower limb amputation proximal to the Symes level among 41 patients was the focus of the prospective study conducted by Kay *et al.* (1987). They found that, compared with patients with a higher number of lymphocytes, patients with a normal lymphocytic count who underwent lower extremity amputation were less likely to experience complications related to healing (p=0.05). Meanwhile, in a different study on 103 patients with diabetic foot ulcers, it was determined that leucocytosis (number of white cells > 11,000 cells/μL) was the only relevant marker of slow rate of healing and the only parameter that could be used in both univariate and multivariate analyses (multivariate odds ratio 9.7, 95% CI 1.0 to 92, p=0.048) (Fleischer *et al.* 2011). The systematic reviews of diabetic foot infection that were undertaken by Lipsky *et al.* (2006) and Zgonis *et al.* (2005) produced similar results. In another study, the determinants of the clinical outcome of below-knee amputation among diabetic foot patients during the period January 2006 – January 2010 constituted the focus of Wong *et al.* (2013). They noted that markers of infection such as high C reactive protein, erythrocyte sediment rate, neutrophils were significantly associated (p=0.01) with poor clinical outcome (good clinical outcome being defined as one not requiring proximal re-amputation or whose stump healed well within 6 months). Mortality rate was 21.2% within 6 months of operation, with sepsis being the most significant cause of death in their study.

5.9 Clotting factors (Prothrombin Time, International Normalised Ratio) and stump healing

The clotting markers namely Prothrombin Time and the International Normalised Ratio in this study were not found to have a significant impact on lower limb stump healing both in univariate as well as multivariate analysis. However, Hasanadka *et al.* (2011) who carried out a study in 4250 (2309 below knee amputations and 1941 above knee amputations) patients over a 3 year period concluded that for lower limb amputations, increasing elevation in International Normalised Ratio predicted a higher wound incidence in the stump (OR = 1.5, p=0.024) and suggested normalization of the International Normalised Ratio prior to the surgery decreases wound incidence in the stump. However, this study was based on the National Surgical Quality Improvement Program (NSQIP) database which according to LaMuraglia *et al.* (2009) may not reflect the cross-section of patients or accepted treatment in present practice. Monroe *et al.* (2012) proposed that the general wound healing response likely depended on a strong initial coagulation response followed by effective deposition of fibrin. Furthermore, given that the initial inflammatory response was inadequate when haemostasis was dysfunctional, they suggested that normal wound healing required ongoing haemostasis. Meanwhile, Roy-Chaudhury *et al.* (2006) highlighted that, aside from promoting proliferation of vascular intima, platelet activation from endothelial damage after an inflammatory reaction also contributed significantly to promotion of platelet aggregating agents like platelet-derived growth factor and thromboxane A_2. A haematoma in the stump following a lower limb amputation is a known complication which acts as a focus for infection and can create dead space, weakening the suture line and thus increasing tension in the wound resulting in impaired healing (Baxter, 2003).

It is standard practice to attempt surgery ideally when the International Normalised Ratio is normalised to prevent excessive bleeding peri and post procedure. To prevent haemorrhaging complications, warfarin-based anticoagulation must be withheld 4-6 days prior to invasive procedure. Even so, high International Normalised Ratio values are still exhibited by some patients on the day of the procedure, which may be postponed, depending on the policy of different centres. Elimination of vitamin K antagonists and the liver's ability for synthesis of coagulation factors II, VII, IX and X determine prothrombin time normalisation, expressed as International Normalised Ratio, a marker restoration of haemostasis (Schwarz *et al.* 2006). In this study, 57.7% (n=173) were noted to have a normal Prothrombin Time out of which 62.4% (n=81) healed their stump and 31.3% (n=94) were noted to have an abnormal International Normalised Ratio out of which 59.6% (n=56) healed their stump.

This study did not take into account the medications that the patients were on including any anticoagulants. Most of the patients had cardiac/stroke related problems and were on anticoagulants including Warfarin. However, before any major surgery as is the usual protocol, all anticoagulants are withheld for at least a period of 3 days or more.

5.10 Lipid profile (total cholesterol, serum triglycerides, serum High Density Lipid cholesterol) and stump healing

Many studies have reported hypercholesterolaemia as a risk factor for lower limb amputation surgery. In our patient population, well established pro-atherogenic factors; hypercholesterolaemia, triglycerides, and High Density Lipoproteins influenced stump healing. Of the 300 patients, 64.7% (n=194) having cholesterol level equal or below 5

mmol/L healed their stump. Low High Density Lipoproteins cholesterol (below 1.1 mmol/L) was noted in 111 patients of whom 56.8% (n=170) healed. The healing rate was 68% (n=204) in patients who had a normal serum High Density Lipoproteins cholesterol (above 1.1 mmol/L). In this study, serum High Density Lipoproteins cholesterol was found to be a predictive marker for lower limb stump healing both in the univariate as well as the multivariate analysis. Patients with a normal serum High Density Lipoproteins had a 75% likelihood of stump healing, in contrast to the patients with an aberrant serum High Density Lipoproteins cholesterol (OR 1.753; 95% CI 1.061 to 2.895; $p<0.026$).

This study however did not take into account the anti-lipid medications which the patients were on both in the retrospective as well as the prospective parts. The cardiovascular risk factors optimisation was not looked into in this study. This is one of the limitations of the study.

Few studies have looked at lipid profile as a marker for stump healing. However, the role of anti-lipid therapies and their effect on lowering the lipid profile leading to a decreased risk in cardiovascular mortality and morbidity is well document. The STENO-2 trial (Gæde *et al.* 2008) which had two arms namely intensive multifactorial intervention against conventional treatment showed that there was a 50% reduction in microvascular and macrovascular events in the intensive arm which included a target of total cholesterol below 175 mg/dl, and triglycerides below 150 mg/dl. In another multivariable analysis, Suckow *et al.* (2012) who studied 436 patients with lower limb amputations between 2003 and 2008 found that the patients most likely to remain ambulatory after a lower extremity amputation were those with preoperative statin use. Similar findings were also reported by Lazzarini *et al.* (2012) who studied one hundred and eighty-six lower limb amputations in 2006-07 and Lee *et al.* (1993) who carried out a retrospective study on Oklahoma

Indians with non-insulin dependent diabetes mellitus (n=1012) who underwent lower limb amputation between 1972-1980. Chaturvedi *et al.* (2001) who reviewed risk factors, ethnic differences and mortality associated with lower-extremity gangrene and amputation in diabetes mellitus in 3443 subjects noted serum triglycerides to be an independent risk factor for lower limb amputation.

In contrast to the findings by the above studies, the Heart Protection Study (2003) which randomly assigned 5963 people with diabetes mellitus to either simvastatin or placebo, showed no difference in amputation rates between the groups for leg amputation (67 [2·2%] vs 67 [2·2%]), or leg ulcer (40 [1·3%] vs 46 [1·5%]), despite substantial reductions in total cholesterol and Low Density Lipoprotein cholesterol concentrations and modest changes in triglyceride and High Density Lipoprotein cholesterol concentrations in the intervention group compared with controls. Rajamani *et al.* (2009) who carried out the Fenofibrate Intervention and Event Lowering in diabetes mellitus (FIELD) study, a pre-specified analysis of a randomised controlled trial with 9795 patients concluded that lipid profile or statin therapy did not alter the risk of a major amputation or effect stump healing (HR 0·93, 0·53–1·62; p=0·79). Nevertheless, in comparison to the placebo group, the fenofibrate group was less likely to require primary non-traumatic amputation, among the 115 diabetic patients with atleast one non-traumatic amputation (HR 0·64, 95% CI 0·44–0·94; p=0·02); implying that fenofibrate was associated with a lower risk of minor amputation than the placebo (HR 0·54, 0·34–0·85; p=0·007). The authors argued that better regulation of lipid profile might not be the only effect of fenofibrate therapy with regard to amputation risk. They based this argument on the observation that multivariable analyses did not indicate any correlation between lipid variables and amputation risk, meaning that lipids did not underpin the influence of fenofibrate in diminishing the risk

of amputation in the Fenofibrate Intervention and Event Lowering in diabetes mellitus study. Correlation of fenofibrate with better endothelium vascular activity (Rosenson *et al.* 2009) and with diminished endothelial dysfunction and pro-inflammation markers (e.g. tumour necrosis factor α, interleukin, and interleukin 1β in plasma) (Ryan *et al.* 2007) have been suggested as potential mechanisms shedding light on the positive effects of fenofibrate on microvasculature. Koh *et al.* (2005) reported that flow-mediated dilator response to hyperaemia, adiponectin levels and insulin sensitivity were all favourably influenced by fenofibrate in patients with hypertriglyceridemia or metabolic syndrome. However, the lack of a standardised routine assessment at baseline to establish vascular status constituted a significant drawback of the Fenofibrate Intervention and Event Lowering in diabetes mellitus study. This raises the possibility of erroneous classification of some amputations due to non-detection of macro-vessel disease as a result of undisclosed angiograms or vascular studies.

5.11 Validation of the prognostic model of stump healing in lower limb amputation

The accuracy of the regression model prognosis depends on model assumptions. The more these assumptions are satisfied, the more accurate the prognosis will be. Nonetheless, experimental data does not allow the complete fulfilment of the assumptions; as such, assessment is geared towards the accuracy of prognosis of a model regarding the information related to new patients. Authentication plays a significant role in providing patients with a reliable appraisal of performance, comparable to the ones obtained in the development sample. Measuring the efficiency of a predictive model for new patients is hence, essential. The process of external validation entails the estimation of prognoses generated by an earlier developed model and their validation using new information, unrelated to the development sample.

The purpose of a predictive model is to assess potential risks and to manage treatment accordingly, in this case, to ensure successful stump healing. This practice can help to structure the process, ensure efficient management of resource distribution, and limit expenses. The ultimate objective is to expand the number of successfully performed lower limb amputation surgeries.

The long-term objective of this research effort was to identify factors predicting lower limb stump healing. The purpose of this study was to develop and validate a prognostic model in the prediction of a successful stump healing. We hypothesized that blood and patient factors could be used to stratify risk of a lower limb stump's failure to heal. In brief, using the development dataset of 300 subjects, we identified three variables associated with lower limb stump healing: serum sodium, serum creatinine and serum High Density Lipoprotein cholesterol, and these variables were validated by using validation dataset of 100 subjects.

Our prognostic model performed well in the external validation of stump healing that involved patients who had not experienced previous lower limb amputation surgery. The performance of the developed model in this study was assessed by discrimination and calibration of the model. The area under the Receiver Operator Curve for a prognostic model is classically between 0.6 and 0.85 (Royston *et al.* 2009). In our study, Receiver Operator Curves was primarily designed for prognostic models, rather for diagnostic models. Receiver Operator Curve was 0.61 in the development model and 0.59 in the validation stage, meaning that the model had reasonable capacity to correctly distinguish between stumps that healed and stumps that didn't. In other words, in a randomly selected patient the outcome would be 59% more likely to have an increased prognosticated probability than a randomly selected patient without the outcome. Ideally, the closer the

ROC is to 1.0, the better the ability to discriminate between the outcome. The researchers understand that the ability to discriminate between stump healing is not ideal. For clinical practice, providing insight beyond the *c* statistic has been a motivation for some recent measures, especially in the context of extension of a prediction model with additional predictive information from a biomarker or other sources (Cook, 2007; Pencina *et al.* 2008)

Accuracy of the model was assessed by examining calibration (Grzegorczyk-Martin *et al.* 2012). To assess the validity of the predictive model developed using the development dataset, we applied the model to an independent or a validation dataset composed of 100 subjects. There was reasonably good agreement between the predicted and observed percentage in predicting stump healing**.** We further note that a substantial size will be required for a validation sample to quantify validity in a reliable way, that is, with enough power to substantial decrease in discriminative ability (Steyerberg *et al.* 2004). In statistical language, the larger the sample size the more robust the results. The ideal sample size is a matter of discussion for any study. Several studies including that of Palazón-Bru *et al.* (2017) have proposed an algorithm to calculate the sample size best suited to externally validate a scoring system. However, the algorithm is complex and does not suit all research designs.

CHAPTER 6

CONCLUSIONS

This chapter provides a brief summary of the study, relates the findings with their implications, discusses the limitations of this study and suggests possible directions for future studies.

6.1 Conclusion of the study

Successful stump healing is a pre-requisite for ambulation following a lower limb amputation surgery. This thesis has provided a detailed evaluation of the risks conferred by some of the key elements on lower limb stump healing. In order to identify which lower limb stumps would heal following a major lower limb amputation surgery, a pre-surgery prediction rule was formulated and verified. This was done in two phases; the development stage and the verification stage. The initial model was developed using data from 300 patients who underwent major lower limb amputation surgery from 2006 to 2009 in the Royal Infirmary of Edinburgh. Three markers namely serum sodium, serum creatinine and serum High Density Lipids cholesterol were recognized as being important predictors of lower limb stump healing. In the second phase these finding were confirmed using data from 100 patients from 2010 to 2011 who underwent major lower limb amputation surgery in the Royal Infirmary of Edinburgh. Due to time restrictions, assessments were carried out pre-operatively and post-operatively without a further follow up assessment, which could provide information on the longer-term consequences. However, additional assessment of the clinical benefits of such a risk classification system in relation to stump healing, based on a larger sample, is necessary. It is of considerable importance that many of the issues described in this thesis continue to be explored, solutions developed, and outcomes improved in this especially large population of vulnerable patients. The safe and effective provision of successful healing of the stump following a lower limb amputation surgery therefore remains an area in which considerable improvements may be made in

the future.

6.2 Clinical implications of research

The results of this study may suggest a number of clinical implications.

In cases where vascular reconstruction is not feasible or is hindered by the configuration of the blood vessels, an efficiently conducted amputation may be a viable solution. In general, the main purpose of an amputation is to restore limb function with the use of prostheses. To this end, as well as to prevent further surgical interventions, it is of the utmost importance to accurately assess the amputation level. However, there are currently no reliable standards that can be referred to prior to leg amputation surgery, even though nowadays, the procedure is carried out on a regular basis. The surgeon has to rely on his clinical judgment and other investigatory parameters including a pre-operative angiogram which has a major role in determining the level of amputation. A meta-analysis performed by Koelemay *et al.* (2001) demonstrated that Magnetic Resonance Angiography (MRA) was highly accurate in diagnosing >50% stenosis or occlusion of arteries in the lower extremity (Level IIa) and played an important part in decision making regarding the site of amputation. However, according to Gu (2004) who looked into the role of an angiogram in 250 amputees, it is not an entirely reliable tool and should be used only as one of the factors rather than the only factor to determine the level of amputation. This study makes this complex decision making easier and adds to the lists of the markers which can play a role in stump healing.

This study has implication in the post amputation rehabilitation process. The amputation level dictates how successful the restoration of limb function will be. The greater the loss, the more is the prosthetic substitution required. Continuing improvements in prosthetic

design cannot substitute for the advantages of a low level of amputation. Retention of the knee is especially important. A functional knee often will allow an elderly person to walk, whereas he or she could not do so with an above-the-knee prosthesis. It is, therefore, of the utmost importance that the surgeon be able to assess accurately the viability of the limb so that amputation can be performed at the lowest reasonable level. Within its parameters this study concludes that if a patient has a normal serum sodium, serum creatinine and serum High Density Lipids, his/her stump is more likely to heal compared to a patients with abnormal serum sodium, serum creatinine and serum High Density Lipids and the chances of needing further revision or an anatomically higher amputation would decrease, resulting in preservation of the knee in a below knee amputation surgery and thereby aiding their rehabilitation. This has an impact not just on the patients but also on the staff involved in the post amputation rehabilitation process including prosthetics, and other allied healthcare professionals.

The level of amputation in lower limb has an impact not just on the patient but also the health system. The three markers which the study has shown to be of significance namely serum sodium, serum creatinine and serum High Density Lipids are blood markers that are routinely done on patients in a clinical setting. The total cost of conducting the above mentioned test is nominal thereby making these markers a cost effective tool to determine stump healing.

Taylor *et al.* (2005) noted that, in the near future, the challenge which both vascular surgeons and patients will be confronted with would be related to the financial and practical aspects of the staged method of limb preservation, which can require numerous surgical interventions and subsequent open bypass, re-do bypass, costly wound treatment, toe amputations, a whole foot amputation, below-knee and then above-knee. Given the

complexity of the staged method of limb preservation, a primary amputation may be a more beneficial solution for the patient. The number of revascularizations could be limited if the stump healing rate prediction was more accurate. The results of this study therefore, are a step forward towards finding an answer about stump healing and the need for revascularization procedures.

6.3 Limitations of the study

There are a number of limitations of this study. Patients were followed up for only 12 weeks in the prospective study due to time restrictions. The formulation of the prediction rule relied on 400 lower limb amputation, constructed in the same medical centre in Scotland. It is possible that the recorded incidence of diabetes mellitus, peripheral vascular disease, and smoking is not illustrative of all the patients with advanced atherosclerosis in Scotland. Hence, this model being a single centre study, did not encompass all the differences in patient demographics and heterogeneity. The prevalence of diabetes mellitus/peripheral vascular disease/smoking in the derivation set may also not be representative of the Scotland amputee population. In the derivation set, surgeons with considerable experience carried out the amputations. However, even with extensive case-mix adjustments seen in previous studies, certain factors which cannot be quantified, including surgical techniques and concepts of care, may be important (Pisoni *et al.* 2002).

One of the major determinants of the level of amputation is the vascular supply. This is effectively measured by radiological imaging. This study did not take into account any form of radiological imaging techniques in determining ideal level of amputation and the subsequent stump healing.

According to Kern *et al.* (2006), lower limb amputations are associated with a number of

risk factors, including chronic kidney disease and peripheral neuropathy. Most of the subjects included in this study had multiple co-morbidities, the severity of which was hard to quantify. The extent of some of the co-morbidities including the duration, control and extent of complications of diabetes mellitus and the extent of renal impairment were not taken into consideration. The impact of medication on stump healing including anticoagulation therapy (aspirin versus. warfarin) was not looked into. Surgical factor such as intraoperative heparin which may play an important role on the surgical outcome were not considered in this study (Feldman *et al.* 2003). The functional effect of multiple failed revascularizations which most of the patients underwent before an amputation was also not taken into account. Other risk factors for amputation such as depression (Tseng *et al.* 2007) were not considered in this study. It is impossible to depict every potential case and result from lower limb amputation surgery with the use of computer simulation models as they can only generate simplified representations.

6.4 Future research

This study can have several follow-up studies. One of them could be the role of endothelial dysfunction markers in chronic limb ischaemia with relation to the haematological markers. Some of the endothelial dysfunction markers including Endothelin-1, von Willibrand factor, Vascular Endothelial Growth Factor (VEGF) and homocysteine, have been shown to be useful predictors to determine healing rates and death events in patients who underwent lower limb amputation (McLaren *et al.* 2002; Newton *et al.* 2005; Groeneweg *et al.* 2008; Newton *et al.* 2008). Haematological markers like preoperative haemoglobin which might be a useful predictor for lower limb stump healing could be looked at. Lee *et al.* (2009) suggested that, prior to vascular surgery, it may prove useful and practical from an economic viewpoint to test and decolonize patients for infection,

particularly over a variety of strains of *methicillin-resistant Staphylococcus aureus* (MRSA). Though this study looked into wound cultures and infection secondary to its retrospective nature, the data obtained was limited and can be looked into by future prospective studies. Dowsett *et al.* (2004) noted that use of prophylactic antibiotics boosts the defense mechanism of patients having limb amputation surgery. Prophylactic antibiotics and its role in stump healing can be looked into on a larger scale. The rehabilitation post amputation plays a vital role in determining the ability of an amputee to walk (Brunelli *et al.* 2006). A follow up of this study could be on the factors that influence the rehabilitation outcomes in lower limb amputation. The impact of early mobility on stump healing could also be explored.

Role of glycated haemoglobin which is an indicator for diabetic control has been investigated (Adler *et al.* 2010). However, the role of insulin vs oral hypoglycaemic agents (OHA) and glycated haemoglobin and its impact on stump healing is a subject for future research. Radiological investigations (radio-nucleotide scans, Magnetic Resonance Imaging) and its effect on stump healing can also be a topic for future research (Croll *et al.* 1996).

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APPENDIX I: NHS ETHICS COMMITTEE APPROVAL

Lothian NHS Board

South East Scotland Research Ethics Committee 2
Waverley Gate
2-4 Waterloo Place
Edinburgh
EH1 3EG
Telephone 0131 536 9000
Fax 0131 536 9088

www.nhslothian.scot.nhs.uk

NHS Lothian

Dr Suhel Ashraff
PhD student
Queen Margaret University, Edinburgh
School of Health Sciences
Queen Margaret University
Queen Margaret University Drive
EH21 6UU

Date 27 July 2011
Your Ref
Our Ref

Enquiries to Lyndsay Baird
Extension 35673
Direct Line 0131 465 5673
Email lyndsay.Baird@nhslothian.scot.nhs.uk

Dear Dr Ashraff

Study title: **FACTORS PREDICTING LEG AMPUTATION SUCCESS IN DIABETIC PATIENTS WITH ADVANCEDATHEROSCLEROSIS.**

REC reference: **11/AL/0305**

Thank you for your letter of 14 July 2011, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a sub-committee of the REC. A list of the sub-committee members is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.
Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

NHS Lothian

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Other conditions specified by the REC –

- ***"What will happen if i take part?" paragraph of Participant Information Sheet should be the same format as the rest of the document. Removed (1) and the indent.***
- ***"Further down the paragraph "assessment will be carried out" should end after" period of assessment." as the last phrase is repetitive.***

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Confirmation should also be provided to host organisations together with relevant documentation.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Evidence of insurance or indemnity		13 August 2010
GP/Consultant Information Sheets	3.1	11 May 2011
Other: Letter from Statistician		19 July 2010
Other: Supervisors CV		
Participant Consent Form	3.1	11 May 2011
Participant Information Sheet		14 July 2011
Protocol	3.1	11 May 2011
REC application	3.1	11 May 2011
Response to Request for Further Information		14 July 2011

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

NHS Lothian

Copy to: *Dr Fiona Coutts*
Dr Tina McLelland, R&D NHS Lothian

South East Scotland Research Ethics Committee 02

Attendance at Sub-Committee of the REC meeting

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mr Thomas Russell	Consultant Neurosurgeon	Yes	
Professor Lindsay Sawyer	Retired	Yes	

NHS Lothian

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

11/AL/0305	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Mr Thomas Russell
Chair

Email: lyndsay.baird@nhslothian.scot.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

"After ethical review – guidance for researchers"

Lothian NHS Board

Edinburgh
EH1 3EG
Telephone 0131 536 9000
Fax 0131 536 9088

www.nhslothian.scot.nhs.uk

NHS Lothian

Dr Suhel Ashraff
School of Health Science,
Queen Margaret University,
Queen Margaret University drive
EH21 6UU

Date 10 September 2010
Your Ref
Our Ref

Enquiries to Lyndsay Baird
Extension 35673
Direct Line 0131 465 5673
Email lyndsay.baird@nhslothian.scot.nhs.uk

Dear Dr Ashraff

Study Title: **FACTORS PREDICTING LEG AMPUTATION SUCCESS IN DIABETIC PATIENTS WITH ADVANCED ATHEROSCLEROSIS.**

REC reference number: **10/S1103/52**

The Research Ethics Committee reviewed the above application at the meeting held on 08 September 2010.

Ethical opinion

The members of the Committee present decided that it was unable to give a favourable ethical opinion of the research for the following reasons:

The Committee noted that the study aimed to assess blood markers and factors in predicting leg amputation success in diabetic patients with advanced atherosclerosis. The results of the study would help the NHS financially, improve the quality of life of patients and also help the decision making of the health professional involved.

The main ethical issues related to the lack of essential information within the IRAS form, whether new tissue was being collected or not and the lack of informed consent to access participant's notes. Overall it was not clear what the Researcher how and what doing with this study. The Committee recommend that he contact the Clinical Care Team and get them to do an retrospective audit prior to resubmitting the other part of the application to the Committee. Dr Bailey agreed to contact the Researcher to assist him with the resubmissions of his application.

The members of the Committee present decided that it was unable to give a favourable ethical opinion of the research, for the following reasons:

- The filter page of the IRAS form has not been completed correctly. New tissue sample question should be ticked and outlined in the participant information sheet.
- Consent to access participant notes has not been obtain as the researcher was out with the Clinical Team.
- It is not clear in the application what comparison were being made between the retrospective and prospective parts of the study.
- The participant information sheet and consent form should be on NHS headed paper not Queen Margaret University College.
- Evidence to support the current factors had not been provided.

NHS
Lothian

Options for further ethical review

You may submit a new application for ethical review, taking into account the Committee's concerns. You should enter details of this application on the application form and include a copy of this letter, together with a covering letter explaining what changes have been made from the previous application. *Other studies submitted direct to RECs:* We recommend that the application is submitted again to this Committee, but you may opt to submit to any another Research Ethics Committee within this domain.

Alternatively, you may appeal against the decision of the Committee by seeking a second opinion on this application from another Research Ethics Committee. The appeal would be based on the application form and supporting documentation reviewed by this Committee, without amendment. If you wish to appeal, you should notify the relevant Research Ethics Service manager (see below) in writing within 90 days of the date of this letter. If the appeal is allowed, another REC will be appointed to give a second opinion within 60 days and the second REC will be provided with a copy of the application, together with this letter and other relevant correspondence on the application. You will be notified of the arrangements for the meeting of the second REC and will be able to attend and/or make written representations if you wish to do so.

The contact point for appeals is:

Joan Kirkbride
Head of Operations
National Research Ethics Service
C/o Janet Kelly
Darlington Primary Care Trust
Dr. Piper House
King Street
Darlington
Co. Durham
DL3 6JL

Tel: 01325 746167
Mobile: 07979 806425
Email: joan.kirkbride@nres.npsa.nhs.uk

Documents reviewed

The documents reviewed at the meeting were:

Document	*Version*	*Date*
Investigator CV	1	11 August 2010
Protocol	1	11 August 2010
Academic Supervisor CV		11 August 2010
REC application	3.0	11 August 2010
Letter of invitation to participant	1	11 August 2010
GP/Consultant Information Sheets	1	11 August 2010
Participant Information Sheet	1	11 August 2010

NHS Lothian

Participant Consent Form	1	11 August 2010
Evidence of insurance or indemnity		13 August 2010
Letter from Statistician		19 July 2010

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

Here you will find links to the following:

a) *Providing feedback: You are invited to give your view of the service you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website*
b) *Re-submission/Appeal*

10/S1102/52	***Please quote this number on all correspondence***

Yours sincerely

Professor Peter Hayes
Chair

Email: lyndsay.baird@nhslothian.scot.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

APPENDIX II: PARTICIPANT DETAILS & HISTORY

Queen Margaret University
EDINBURGH

PARTICIPANT CONTACT DETAILS

Study No.	
First Name	
Surname	
D.O.B.	
Address	**Postcode:**
Tel.	
Mobile	
Email	

Queen Margaret University

EDINBURGH

Study No.	

MEDICAL HISTORY	
Age	
Gender	
Duration of Disease	
Risk Factors/Co morbid diseases	□ Diabetes □ Hypertension □ Hypercholesterolemia □ Smoking
Symptoms	

MEDICATIONS		
Drug Name	**Dosage**	**Frequency**

Queen Margaret University

EDINBURGH

Study No.	

Baseline markers	
Date	
D.O.B.	
BP	
HbA1C	

Blood Markers	
White cell count	
C-reactive protein	
Urea	
Serum Creatinine	
INR	
Prothombin time	

Serum Sodium	
Serum Potassium	
Total Cholesterol	
Triglyceride	
HDL	
LDL	

Queen Margaret University
EDINBURGH

Study No.

Surgery Date:
Type of Surgery: **Below knee amputation** ☐
Has the stump healed/fit enough to take a prosthesis at 12 weeks? **Healed** ☐ **Not healed** ☐

APPENDIX III: PARTICIPANT INFORMATION SHEET

Participant Information Sheet

"Can biomedical markers play any role in predicting success of leg amputation in diabetic patients with advanced atherosclerosis?"

My name is Suhel Ashraff and we would like to invite you to take part in a research study examining the role of biomedical markers and factors in predicting success of leg amputation in patients with atherosclerosis and Diabetes.

Before you decide if you would like to participate, you need to understand why the research is being done and what it would involve for you. ***Please take time to read the information carefully***. Talk to others about the study if you wish.

Please feel free to ask me if anything is not clear or if you would like more information. My contact details are at the end of this brochure.

What is the purpose of this study?

The purpose of the study is to explore the relationship between the blood markers and factors (clotting factors, cholesterol and blood sugar) and success of leg amputation. It will examine the consistency of one assessor with the outcome measure as well as reliability between two different assessors.

Why have I been asked to take part?

You have been asked to take part as you have previously been diagnosed with atherosclerosis and you are due to have a lower limb amputation.

Do I have to take part?

No, it is up to you to decide whether or not to take part and your participation in this research is entirely voluntary. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. Deciding not to take part or withdrawing from the study will not affect the healthcare that you receive.

What will happen if I take part?

Once your consent is obtained you will be assessed on two separate occasions. Each visit for assessment will be approximately 20 minutes. Your visit plan will be as follows:

1) Visit 1: Evaluation prior to surgery/pre-operative. This will happen when a decision has been made that you will have a lower limb amputation and you are admitted to the ward. Alternatively this could be your last clinic appointment prior to the date of lower limb amputation surgery.

2) Visit 2: 12 week's later/post-operative. Your stump will be assessed. This will happen in you clinic appointment.

Both the assessors are familiar with the procedure, one being myself and the other vascular surgeon.

At the time of your assessments, the following will happen:

At each visit, you will have the chance to ask questions and raise any issues before, during or after each assessment.

↓

Your blood sample *(1)* will be taken (only for the initial visit). This will take approximately 20 minutes

For the assessment, you will have to remove your jacket so that your clothing does not interfere with the procedure. The amount of blood taken will be 2 teaspoon full (about10 mls). Blood sample will be taken by NHS staff as a regular procedure for pre-operative tests only in the first visit.

There are no known side-effects associated with either of these assessments.

(1) *For the biomedical markers analysis blood sample will be taken. From these test we will calculate your kidney function status, infection markers and lipid profile as mentioned above (these are routine tests done in the hospital).*

With your consent we will inform your GP that you are taking part in this study.

The blood will be stored in the laboratory in the Royal Infirmary of Edinburgh in an anonymised form and the donor will not be identifiable to the researcher.

Will there be any disadvantages of taking part?

It is not thought that there are many disadvantages; however, it is possible that you might develop a bruise from the site of blood withdrawal. If this was to happen you will be treated appropriately. Taking part in this study will not put you at risk of any bodily or mental harm. Your health status will not be altered in anyway by taking part.

Assessments will be carried out with respect for your privacy and your comfort will be ensured before and during the entire period of assessment to minimise any discomfort.

What are the benefits of taking part in the study?

You may learn about your body composition.

What will happen to the results of the study?

If you are interested in the overall results of the study, these will be emailed to you as well once the study is complete.

The results may be published in a journal or presented at a conference or used as a part of a PhD thesis. Your anonymity will be preserved.

What happens when the study is finished?

At the end of the research we will inform you about the results of the study through written feedback. The data will be kept in a safe in Queen Margaret University for no longer than 12 months the access to which will be restricted to the Chief Investigator.

Will my taking part in the study be kept confidential?

All information about you collected during the course of the study will be kept securely and will be accessible only to the research team members and there are strict laws which safeguard your privacy at every stage.

All information, which is collected, about you during the course of the research will be kept strictly confidential.

All data will be anonymised and your name will be removed from the data so that you cannot be recognised from it.

What will happen to the results of the study?

The study will be written up as a PhD thesis and the results may be published in a journal or presented at a conference.

Who is organising the research?

This study has been organised by Queen Margaret University, Edinburgh and is self funded by the Chief Investigator.

Who has reviewed the study?

This study has been reviewed and given favourable ethical opinion by the Queen Margaret University Research Ethics Committee and South East Scotland Research Ethics Committee 2. NHS management approval has also been obtained.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. If you have a concern about any aspect of this study, you could ask to speak to me and I will do my best to answer your questions (Queen Margaret University telephone: 01314740000). Alternatively, you could also contact the NHS Lothian Complaints Team (contact details given), if you should wish to complain formally.

What if I do not want to continue my participation?

You are free to withdraw from the study at any given point without having to give a reason for doing so.

If you have read and understood this information sheet, any questions you had have been answered, and you would like to be a participant in the study, please now see the consent form.

Further information and contact details

Name of researcher: Suhel Ashraff

Address: Postgraduate Research Student, Podiatry
School of Health Sciences
Queen Margaret University
Queen Margaret University Drive,
Musselburgh,
EH216UU

Email / Telephone: drsuhelashraff@gmail.com / 0131 474 0000 (ext 4796)

If you would like to discuss this study with someone independent of the study please contact:

Name: Mr J. Murie
Post: Consultant Vascular Surgeon
Royal Infirmary of Edinburgh
Little France, Old Dalkeith Road
Edinburgh
EH16 4SA
Email / Telephone: jmurie@qmu.ac.uk / 0131 537 1000

If you wish to make a complaint about the study please contact NHS Lothian:

NHS Lothian Complaints Team
2nd Floor
Waverley Gate
2-4 Waterloo Place
Edinburgh
EH1 3EG
Tel: 0131 465 5708

Thank you for taking the time reading this information sheet.

APPENDIX IV: CONSENT FORM

CONSENT FORM

Title of Project:
"The Role of haematological markers and factors in predicting leg amputation success in diabetic patients with advanced atherosclerosis: An Exploratory Study"

Please initial box

1. I confirm that I have read and understood the information sheet and this consent form. ☐
   I have had an opportunity to ask questions about my participation.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, ☐ without giving any reason, without my medical care or legal rights being affected.

3. I understand that sections of any of my medical notes may be looked at by responsible ☐
   Individuals (Research Team) or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

4. I agree to take part in the above study. ☐

5. I give consent to contact my G.P to inform him that I am taking part in this study. ☐

_____	_____	_____
Name of Patient		Date Signature

_____	_____	_____
Researcher	Date	Signature

Contact details of the researcher

Name of researcher: Dr Suhel Ashraff

Address: PhD Student, Podiatry, School of Health Sciences

Queen Margaret University

Edinburgh, EH21 6UU

Email / Telephone: drsuhelashraff@gmail.com / 0131 474 0000

1 for patient; 1 for researcher; 1 to be kept with hospital notes

APPENDIX V: G.P LETTER

Queen Margaret University
EDINBURGH

Suhel Ashraff
MBBS, MSc. Diabetes
PhD Research Student
School of Health Sciences
Queen Margaret University
Queen Margaret University Drive
Musselburgh
EH21 6UU
Tel 0131 4740000

Date:

Dear Dr .....

Re: ..... D.O.B. .....
Address: .....
.....
.....
This patient has agreed to take part in the following research project:

"The role of haematological markers and factors in predicting leg amputation success in diabetic patients with advanced atherosclerosis: An Exploratory Study"

The study is being carried out at the New Royal Infirmary in Edinburgh and at Queen Margaret University in Musselburgh.

I enclose the summary of the protocol for your information.

If you would like more information about this project, please don't hesitate to contact us.

Yours sincerely,

Dr Suhel Ashraff, Research Student, QMU, Edinburgh

Dr Thomas E Carline, Senior Lecturer & Course Director, QMU, Edinburgh

Mr Zahid Raza, Consultant Vascular Surgeon, Royal Infirmary of Edinburgh

Dr Derek Santos, Senior Lecturer, QMU, Edinburgh

APPENDIX VI: LABORATORY DETAILS OF THE BLOOD MARKERS

Biochemistry

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Analyte	**Methods used for measurement**	**Analyzer used**	**Range**	**Calibration**	**Measured at**
Serum Sodium	Indirect ion-selective electrode – all three electrolytes are measured simultaneously within a single 'chip' Potentiometric Ion selective electrode (crown ether membrane)	Abbott Architect c16000	Ref range 135-145 mmol/L	Calibration 66320 ISE Buffer 66319 ISE Mid Standard 66318 ISE Reference 66317 ISE Low serum Std 66316 ISE High serum Std 66314 Internal reference 66313 ISE Na+ / K+ Selectivity check	Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,
Urea	Enzymatic reaction involving urease. The change in absorbance of the solution (caused by the oxidation of NADH) is measured photometrically at	Abbott Architect c16000	Ref Range 2.5-6.6mmol/L	Calibrator Olympus system Calibrator 66300 Kinetiv UV, Cat No OSR6534	Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,

	340nm				
Serum Creatinine	Kinetic alkaline picrate reaction – rate of absorbance change measured photometrically at 500nm. Standard kinetic Jaffe method	Abbott Architect c16000	Ref range 60-120 umol/L	Calibrator Olympus system Calibrator 66300 Kinetic Jaffe, Cat No OSR6178	Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,
CRP	Latex immunoassay whereby agglutination of antigen-antibody complexes causes a change in absorbance at 572nm Olympus System CRP Latex reagent	Abbott Architect c16000	Ref Range 0-5 mg/L	Calibrator (Normal set: ODC 00026) (Highly sensitive set:ODC00027) Cat No OSR6199	Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,
Total Cholesterol	Enzymatic reaction involving cholesterol esterase. Formation of a coloured dye is measured photometrically at 500nm Enzymatic colour test, Cat No	Abbott Architect c16000	No range given	Calibrator - Olympus System Cal 66300	Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,

	OSR6116				
Triglycerides	Enzymatic reaction involving glycerol phosphate oxidase. Formation of a red dye is measured photometrically at 500nm Enzymatic colour test: Cat No OSR6118	Abbott Architect c16000	Ref Range 0.8-2.1mmol/L	Calibrator: 66300	Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,
HDL	Uses a detergent to solubilise HDL, which then reacts with other compounds to form a coloured dye, measured photometrically at 604nm Enzymatic colour test: Cat No OSR6187	Abbott Architect c16000	Ref Range 1.1-1.7 mmol/L	Calibrator HDL cholesterol calibrator ODC0011	Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,
Serum Potassium	Indirect ion-selective electrode – all three electrolytes are measured simultaneously	Abbott Architect c16000	Ref range 3.6-5.0 mmol/L	Calibration 66320 ISE Buffer 66319 ISE Mid Standard	Clinical Laboratory, Royal Infirmary of

	within a single 'chip' Potentiometric Ion selective electrode (crown ether membrane)			66318 ISE Reference 66317 ISE Low serum Std 66316 ISE High serum Std 66314 Internal reference 66313 ISE Na+ / K+ Selectivity check	Edinburgh, Scotland, UK,
LDL	Calculated from the total cholesterol and the HDL cholesterol	laboratory analyzer interface-AMS.	No range given	Calibrator - Olympus System Cal 66300	Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,
eGFR	A calculated parameter, which takes into account serum creatinine, age and sex.	laboratory analyzer interface-AMS.			Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,
	HPLC (reversed-phase cat ion exchange	ADAMS HA-8160 (Menarini	Ref Range (DCCT) % total Hb 4-	Calibrator: Cat No Ref 23385 Low and High	Clinical Laboratory, Royal

HbA1C	chromatography)	Diagnostics)	6	standard	Infirmary of Edinburgh, Scotland, UK,
Creatinine Kinase	Kinetic UV (based on recommendations IFCC), Cat No OSR6179	laboratory analyzer interface-AMS.	Ref Range 55-170 U/L	Calibrator Olympus System Cal 66300	Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,

Analyzer

- The Abbott Architect c16000 is a fully automated, fast throughput chemistry analyzer that can perform analyses by various photometric and potentiometric methods.
- All lab analysis is tightly controlled by various protocols and procedures regarding quality.
- There is a labs-wide quality manual, produced by the laboratories Quality Manager, which sets out what labs must do to conform to the Clinical Pathology Accreditation (CPA) standards that govern the labs.
- The quality control inspection happens every two years.

Calibration and Validation

- Calibration is performed using calibrator solutions provided by Abbott that are traceable to international reference standards.
- All analytes have a manufacturer's calibration interval programmed in the analyzer.
- If the calibration interval is exceeded, it is not possible to use the assay until a calibration is performed.
- Calibration intervals range from 24 hours to a few weeks, depending on the analyte, but calibration may also be required when quality control results start to drift.

Quality control

- Quality control is run for all of the above analytes every four hours and analyzed by Biomedical Scientist staff for any bias or imprecision that may require calibration or other analyzer troubleshooting to resolve.
- If quality control for an analyte is outside the acceptable range or fails preset Westgard rules, all patient results are held back by the analyzer interface until the operator has resolved the problem.
- The lab also participates in external quality assurance schemes for all analytes.
- For those above, the provider of these schemes is UKNEQAS.
- On a fortnightly basis (monthly for lipids and CRP), three samples of unknown concentration are sent out to the lab.
- All three are analyzed and the results are returned to the UKNEQAS, who then run calculations based on the results of all participating labs (across the whole of the UK) and award each lab their own scores relating to accuracy and bias of their results compared with other labs.
- In this way performance of each laboratory is ensured and is comparable to the rest of the UK, or at least to other labs using the same analyzers.
- Any consistent poor performance is notified by the scheme providers by way of a letter, which must be investigated and resolved.

Haematology

Analyte	**Analyzer used**	**Range**	**Calibration**	**Measure at**
White cell count	Sysmex XE 2100 Full Blood Count analyzers (Supplier: Sysmex UK)	Ref Range 4.0 - 11.0(x 109/l)	Calibrations: when Internal Quality control deviates from baseline, poor EQA or after significant service maintenance	Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,
Prothrombin time	Sysmex XE 2100 Instrumentation Laboratory, UK	The normal range: 10.5 - 13.5 seconds.	Reagent: HemosIL Recomboplastin 2G IL UK).	Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,
International Normalised Ration (INR) was (Instrumentation Laboratory, UK) (Reagent: HemosIL Recomboplastin 2G IL UK). Specimens were stored at 24-36 hours.	Sysmex XE 2100 Instrumentation Laboratory, UK		Reagent: HemosIL Recomboplastin 2G IL UK).	Clinical Laboratory, Royal Infirmary of Edinburgh, Scotland, UK,

Calibration

- Calibration is only required if the daily QC falls out with its range value.
- The XE Analyzers are calibrated on Installation, using Factory set CS-1000 calibrator at source, but checked with Sysmex E-Check on site.
- Internal QC is performed using e-Check in the morning.
- The 3 modes of the analyzer (Auto/Manual/Capillary) are checked daily, and aspirate a fresh sample to check WBC and Differential.
- Most of the other tests are calibrated using the IL (manufacturer of analyzer) calibration material.
- This is traceable to WHO standards.
- Calibrations are performed as and when required, for example when a lot of reagent/ material changes.
- The calibration material has assigned values for tests, test protocols are written into the software of the analyzer.
- The mean normal PT is established using healthy volunteers and then the ISI of the reagent is determined using WHO calibrant.
- This is further validated by running Internal QC and previously performed External QC.
- Routine operation of the analyzers involves IQC being performed post daily maintenance procedure and every 6 hours after this or when a vial of thromboplastin is changed.
- Tests cannot be performed if the IQC fails.
- IQC is performed using a normal and abnormal control.
- All necessary reagents/ materials are placed onto the analyser and the calibration is performed.
- The operator must then validate the calibration curve.

Validation

- External QC involves registration with UK NEQAS. Samples for these trials are analysed monthly.
- Analyzer Validation was based upon the MHRA model: MHRA 03058 February 2003
- For reference, the Operation of the Analyzers, and Maintenance of the Analyzers are listed in: HAEM-R-66 (Operation of the Sysmex XE HST System), and HAEM-R-177 (Maintenance of the XE5000 Analyzer).
- All documentation on this is held electronically in a database.
- Each analyzer is validated for PTs/ INRs when a new lot of thromboplastin reagent is introduced, prior to the current batch running out.

Quality control

- Quality control is run for all of the above analytes every four hours and analyzed by Biomedical Scientist staff for any bias or imprecision that may require calibration or other analyzer troubleshooting to resolve.
- If quality control for an analyte is outside the acceptable range or fails preset Westgard rules, all patient results are held back by the analyzer interface until the operator has resolved the problem.
- The lab also participates in external quality assurance schemes for all analytes.
- For those above, the provider of these schemes is UKNEQAS.
- On a fortnightly basis (monthly for lipids and CRP), three samples of unknown concentration are sent out to the lab.
- All three are analyzed and the results are returned to the UKNEQAS, who then run calculations based on the results of all participating labs (across the whole of the UK) and award each lab their own scores relating to accuracy and bias of their results compared with other labs.
- In this way performance of each laboratory is ensured and is comparable to the rest of the UK, or at least to other labs using the same analyzers.
- The precision method used for all the laboratory tests was the Westgard rules wherein the analytical method is first tested under ideal conditions. Following this the analytical

method was then put to use in routine practice as noted under the Westgard rules. Using the Levey Jennings chart.

- Any consistent poor performance is notified by the scheme providers by way of a letter, which must be investigated and resolved.

APPENDIX VII: INDEMNITY

All participants were informed about procedure followed during the study. They were also informed about any possible harm they might suffer and how it would be addressed. If patients had any concern about any aspect of this study, they were given the contact details of the principal researcher. They were also provided with the chief investigators contact details if they had any questions. Alternatively, they could also contact the independent advisor who was aware about the project but was not directly involved in this research (contact details given) or the NHS complaints team, if they wished to complain formally.

Compensation for QMU financed or co-financed medical research was covered by the Royal and Sun alliance insurance, which encompassed all participants in medical tests, together with their relatives, charges, executors, administrators or legal representatives. In general, referrals were not necessary as the insurance policy included wide-range medical research and tests. No complaint was received in this study.

Royal and Sun Alliance insurance provides indemnity for QMU sponsored or co-sponsored clinical study (Appendix IV). This is a no-fault compensation policy and covers any person taking part in a clinical trial including their dependants, executors, heirs, administrators and legal representatives. Cover applies automatically to clinical trials and general clinical research within wide parameters without the need for referral although there are exclusions. In this study no complaints were received.

APPENDIX VIII: INSURANCE LETTER

Chris Faulds
Client Adviser

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christopher.faulds@marsh.com
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Queen Margaret University
Queen Margaret University Drive
Musselburgh
East Lothian
EH21 6UU

11th August 2015

To whom it may concern

Dear Sirs

CONFIRMATION OF INSURANCE – Queen Margaret University, Edinburgh & Subsidiary Companies

As requested by the above client, we are writing to confirm that we act as Insurance Brokers to the client and that we have arranged insurance(s) on its behalf as detailed below:

PRIMARY PUBLIC / PRODUCTS LIABILITY

INSURER:	Royal & Sun Alliance
POLICY NUMBER:	RTT153481
PERIOD OF INSURANCE:	1st August 2015 to 31st July 2016 – both days inclusive
LIMIT OF LIABILITY:	GBP20,000,000 any one occurrence, and in the annual aggregate in respect of Products
DEDUCTIBLE:	GBP1,000 Third Party Property Damage

EMPLOYERS LIABILITY

INSURER:	Royal & Sun Alliance
POLICY NUMBER:	RTT153481
PERIOD OF INSURANCE:	1st August 2015 to 31st July 2016 – both days inclusive
LIMIT OF LIABILITY:	GBP25,000,000 any one event GBP5,000,000 in respect of Terrorism
DEDUCTIBLE:	NIL

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MARSH & McLENNAN COMPANIES

MARSH

This letter may not be reproduced by you or used for any other purpose without our prior written consent.

This letter shall be governed by and shall be construed in accordance with English law.

Yours faithfully,

Chris Faulds
Client Adviser
Marsh Ltd

Hasilwood House
60 Bishopsgate
London EC2N 4AW
Tel: 020 7847 8670
Fax: 020 7847 8689

TO WHOM IT MAY CONCERN

12th September 2016

Dear Sir/Madam

QUEEN MARGARET UNIVERSITY
AND ALL ITS SUBSIDIARY COMPANIES

We confirm that the above Institution is a Member of U.M. Association Limited, and that the following covers are currently in place and that cover is worldwide:-

EMPLOYERS' LIABILITY

Certificate No.	Y016458QBE0116A/174
Period of Cover	1 August 2016 to 31 July 2017
Limit of Indemnity	£25,000,000 any one event unlimited in the aggregate.
Includes	Indemnity to Principals
Cover provided by	QBE Insurance (Europe) Limited and Excess Insurers.

PUBLIC AND PRODUCTS LIABILITY

Certificate of Entry No.	UM174/16
Period of Cover	1 August 2016 to 31 July 2017
Includes	Indemnity to Principals
Limit Of Indemnity	£50,000,000 any one event and in the aggregate in respect of Products Liability and unlimited in the aggregate in respect of Public Liability.
Cover provided by	U.M. Association Limited and Excess Cover Providers led by QBE Insurance (Europe) Limited

If you have any queries in respect of the above details, please do not hesitate to contact us.

Yours faithfully

Susan Wilkinson
For U.M. Association Limited