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Connexin 43 as a biological marker of ischaemia in occlusive arterial disease of the lower limb

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Abstract

Diabetes mellitus is a worldwide health issue. It is a major and growing cause of morbidity and mortality. Rates of end-organ damage are increasing proportionately, as are the associated personal, societal and economic costs. Diabetic foot ulceration is responsible for a significant proportion of this. The development of diabetic foot ulceration is complex with multiple injurious processes potentially affecting a foot at any time. Defining the significance of ischaemia as a consequence of peripheral arterial occlusive disease can be difficult and identifying which patients may benefit from revascularisation in terms of reversing of the natural history of the disease process remains poorly understood.

The utility of the University of Texas classification to identify patients at highest risk of major adverse clinical events has been explored from 971 ulcer episodes affecting 515 limbs (388 patients). Peripheral arterial occlusive disease was identified in 44.6%. These patients had a significantly higher risk of major amputation than patients without peripheral arterial occlusive disease (13.5 versus 4%). University of Texas scoring would appear to robustly identify patients at highest risk of major adverse clinical events.

The potential usefulness of Connexin 43 and its phosphorylated isoform Connexin 43(Serine368) as biological markers of ischaemia in skin have been explored. Immunohistochemistry provided qualitative data demonstrating up regulation of Connexin 43(Serine368) in skin biopsies from ischaemic feet – this is a novel finding. Connexin 43 (Serine368) was not identified in any of the controls nor in proximal skin biopsies of patients with peripheral arterial occlusive disease. Connexin 43 expression did not appear to be modified.

When human fibroblasts and keratinocytes were subjected to hypoxic conditions (1% oxygen/4% carbon dioxide/nitrogen) in vitro, both Connexin 43 and Connexin 43(Serine368) were up regulated with protein expression peaking between 12 to 24 and 6 to 12 hours respectively. Peptidoglycan challenge appeared to up-regulate the expression of Connexin 43 protein, but did not influence the expression of Connexin 43(Serine368). These data suggest that Connexin 43(Serine 368) may have potential utility as a biological marker of ischaemia in human skin

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Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

The statistical analyses in Chapter 2 were completed with assistance from Mr. WP Stuart.

Abbreviations

ABPI	Ankle/Brachial Pressure Index
BSA	Bovine Serum Albumin
cDMEM	complete Dulbecco's Modified Eagle Medium
CAM	Cellular Adhesion Molecule
CLI	Critical Limb Ischaemia
CRP	C-Reactive Protein
CTA	Computed Tomography Angiography
Cx	Connexin
CxHc	Hexameric Connexons
Cx43	Connexin 43
Cx43(Ser368)	Connexin 43 (Serine 368)
DAPI	4'-6-Diamidino-2-phenylindole
DPX	Distyrene, a plasticizer (tricresyl phosphate), and xylene
DFU	Diabetic Foot Ulceration
DM	Diabetes Mellitus
DSA	Digital Subtraction Angiography
FBS	Foetal Bovine Serum
GAPDH	GlycerAldehyde-3-Phosphate Dehydrogenase
GJ	Gap Junctions
HT	Hyperspectral Technology
IL-6	Interleukin-6
LDL	Low Density Lipoprotein
MACE	Major Adverse Clinical Event
MRA	Magnetic Resonance Angiography
mRNA	messenger Ribonucleic Acid
NICE	National Institute of Clinical Excellence
NO	Nitric Oxide
PAOD	Peripheral Arterial Occlusive Disease
PBS	Phosphate Buffered Saline
Revasc	Revascularisation
SPSS	Statistical Package for the Social Sciences
TF	Tissue Factor
TP	Toe Pressure

TBI	Toe/Brachial Pressure Index
UoT	University of Texas Classification
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor
WHO	World Health Organisation
WIfI	Wound, Ischaemia and foot Infection

Glossary of Terms

Ankle Brachial Pressure Index (ABPI) - A ratio of the systolic blood pressure recorded in a tibial artery at the level of the ankle and the brachial artery in the arm

Bradford protein assay - a spectroscopic analytical procedure used to determine the protein concentration

Connexin (Cx) – Structurally related transmembrane proteins which form gap junctions

Cryostat - A cold chamber in which frozen tissue is divided with a microtome

Critical Limb Ischaemia (CLI) – A clinical condition associated with peripheral arterial occlusive disease in which there is threat to the long-term viability of the limb without intervention

Diabetes Mellitus (DM) – A metabolic condition associated with the development of hyperglycaemia as a consequence of a failure of insulin production or the development of insulin resistance

Fibroblast – A cell type that is involved in the synthesis of extra-cellular matrix and collagen. Fibroblasts have an important role in wound healing

Gap junctions – Intercellular connections between adjacent cells

Keratinocyte – The most prominent cell in the epidermis. These cells produce keratin as they undergo terminal differentiation

Peripheral Arterial Occlusive Disease (PAOD) – A manifestation of systemic atherosclerosis, which can result in large vessel arterial obstruction

Phosphorylation - A biochemical process that involves the addition of phosphate to an organic compound. Phosphorylation reactions are mediated by the kinases or phosphotransferases

Toe Brachial Pressure Index (TBI) – A ratio of the systolic pressure recorded in a digital artery in the toe and the brachial artery in the arm

Ulcer – A breach in an epithelial surface

University of Texas (UoT) classification – A scoring system to describe diabetic foot ulceration that assesses ulcer depth and the presence of infection and ischaemia

Western blot analysis – A semi-quantitative analytical technique that can be used to detect specific proteins in a sample of tissue homogenate or extract

Chapter 1 - Definition of critical limb ischaemia and a review of the challenges of diabetic foot ulceration, with discussion of the potential utility of Connexin 43 as a biological marker of ischaemia in patients with atherosclerotic arterial disease of the lower limb

1.1 Introduction

Peripheral arterial occlusive disease (PAOD) of the lower limb is a consequence of systemic atherosclerosis. The Edinburgh Artery Study reported a prevalence of 8% for asymptomatic PAOD in a study population of 1,592 participants (aged 55–74 years), with symptomatic disease (intermittent claudication) diagnosed in 4.6% [Fowkes 1991]. In the symptomatic population group as many as 20% of patients may have a diagnosis of diabetes mellitus (DM) [Murabito 1997].

A small number of patients with severely symptomatic PAOD will develop critical limb ischaemia (CLI) with threat to limb viability. The estimated incidence of CLI in Europe and North America is 500-1,000 per million of the population. At 1 year, approximately 25% of these patients will have died and as many as one-third will have had a major limb amputation performed [Norgren 2007]. There are clear criteria for the diagnosis of CLI [European Working Group on Critical Limb Ischaemia 1992] which include either: More than 2-weeks of recurrent foot pain requiring the regular use of analgesics, associated with an absolute ankle systolic blood pressure of 50mmHg or less (or an absolute systolic toe pressure of 30mmHg or less) or; A non-healing wound or gangrene of the toes or foot, with similar haemodynamic measurements.

Diagnosing CLI in patients with DM and foot ulceration can be difficult and confusing. Frequently a distal pattern of arterial disease (infra-geniculate) is encountered [Rueda 2008]. In the context of neuropathy pain may be absent. These features can be compounded by the presence of arteriovenous shunting conferring a red, hyperaemic colour to the foot.

1.2 Diabetic foot ulceration: A significant and complex problem

DM is a worldwide health issue, it has been estimated that 422 million adults were living with the disease in 2014. It is a major and growing cause of morbidity and mortality [WHO 2016]. In Scotland the prevalence of type II DM is rising; over the next 25 years one in ten people is expected to develop the disease [Scottish Executive 2006].

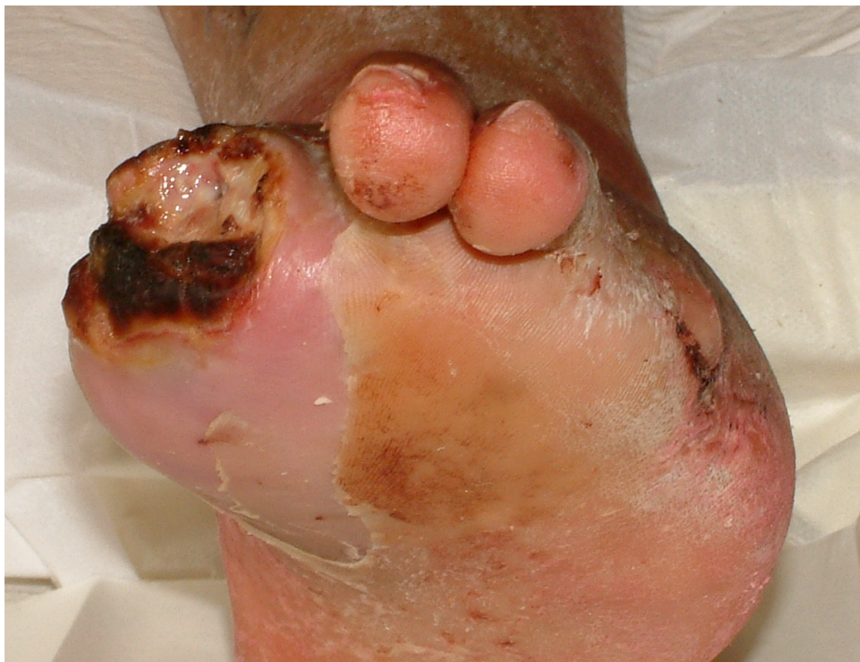
Rates of end-organ damage are increasing proportionately, as are the associated personal, societal and economic costs. The National Health Service currently spends approximately 10% of the healthcare budget (approximately £9 billion per year) on the management of DM and its complications. Diabetic foot ulceration (DFU) is responsible for a significant proportion of this cost. Worldwide it is estimated that 50% of all non-traumatic lower limb amputations are performed for patients with a diagnosis of DM [WHO 2016].

There is wide variation in reported rates of (DFU), probably reflecting ethnicity, social deprivation and case ascertainment [Jeffcoate 2012]. DFU is a relapsing-remitting process that has a high impact on patients, their carers and health care systems [Reiber 1998, Boulton 2005].

The natural history of DFU in the developed world has been well described with a mortality rate of 16.9% and an amputation (predominantly infra-malleolar) rate of 11% at one year [Jeffcoate 2006]. In 25-80% there will be persistence or ulcer recurrence after one year of treatment [Lavery 2007].

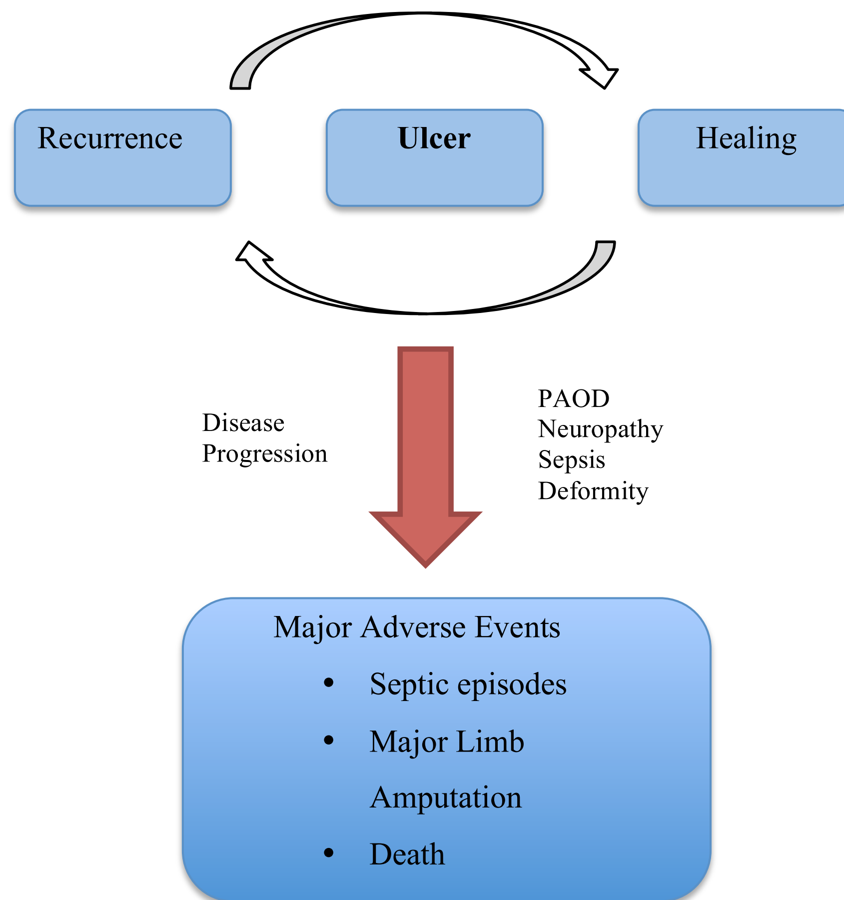
The development and recurrence of DFU is complex with multiple injurious processes potentially affecting a foot at any time. Neuropathy and ischaemia (as a consequence of PAOD and/or endothelial dysfunction, often referred to as microvascular disease) are fundamental components in the development of ulceration – with the term neuroischaemic ulceration describing the clinical state where there is both neuropathy and an ischaemic component (Figure 1.1).

Figure 1.1 Neuroischaemic diabetic foot ulceration
This example eloquently demonstrates the challenges associated with DFU – there has been minor amputation of the fourth and fifth toes with wound healing (without arterial intervention). Subsequent to this there has been amputation of the hallux with wound failure despite arterial reconstruction (femoro-distal bypass). The clinical course for this patient was further deterioration in the foot resulting in major limb amputation (transtibial), with failure to rehabilitate with a prosthetic limb and cardiovascular death within 12-months.



Other important pathophysiological processes include repeated episodes of acute and/or chronic infection, mechanical changes as a consequence of Charcot change or surgical interventions and metabolic dysfunction associated with hyperglycaemia (at a cellular level this may result in impaired Nitric Oxide synthesis [Goldin 2006], structural and functional changes of the fibroblasts [Loots 1999] and an increase in matrix metalloproteinase activity [Vaalamo 1999]) all of which can contribute to tissue damage. These processes can result in a progressive cycle of tissue destruction, septic episodes, and mechanical foot deformity (there is loss of intrinsic muscle mass and no opposition to the powerful flexors, resulting in 'clawing') with subsequent loss of function (Figure 1.2). As a consequence of this a significant proportion of patients with DM will undergo major limb amputation, despite attempts at limb salvage or suffer complications relating to severe sepsis including myocardial infarction, renal failure and death (Jeffcoate 2006).

Figure 1.2: The natural history of diabetic foot ulceration



These features can leave clinicians struggling to know which the most important components in the process are, but perhaps more importantly what the effects of treating or reversing these processes may have. Breaking this cycle requires action against each component: drainage of sepsis and debridement of necrotic tissue with appropriate antibiotic therapy, improved glycaemic control, pressure relief and in appropriate cases restoration of tissue perfusion.

Although ischaemia can undoubtedly slow healing, many DFU will heal without vascular improvement [Elgzyri 2013] even in the presence of PAOD. The median time to DFU healing is reported to be 3-months (2-7months) [Leese 2007, Prompers 2008]. Consequently significant controversy exists within the surgical community regarding the role of very distal and infra-malleolar revascularisation in patients presenting with DFU, given the described multifactorial aetiology and the potential for improvement without revascularisation.

Identification of how significantly ischaemia as a consequence of PAOD may be contributing to DFU can be uncertain with current investigative strategies. Consequently identifying which patients may benefit from revascularisation, in terms of reversal of the natural history of the disease process remains poorly understood despite potentially being central to patient management. This clinical difficulty has been highlighted by systematic review of revascularisation for DFU, which reported a limb salvage rate of 80-85% [Hinchcliffe 2012, with ulcer healing occurring in only 60% (comparable to healing rates without intervention) [Leese 2007, Elgzyri 2013].

The National Institute of Clinical Excellence (NICE) has attempted to address this issue. A simple review question was asked - "when is the optimal time for surgical management (including vascular surgical reconstruction and orthopaedic interventions) to prevent amputation for diabetic foot problems?" A systematic search identified 9817 potentially relevant studies. However none of these met the inclusion criteria; as a consequence no consensus statement could be made [NICE 1993].

To try and define the utility of revascularisation for patients with DFU we must first try to establish the significance of PAOD.

1.3 Assessment of the peripheral arterial circulation of patients with diabetic foot Ulceration

In much the same way as there is variation in the reporting of DFU [Chan 2015], there is variation in patient investigation and management. To be effective evaluation of DFU must be multifaceted. There is evidence demonstrating improved patient outcomes (reduction in both the rate of major limb amputation and the duration of hospital admission) if a multidisciplinary approach is adopted [NICE 2015]. Included in the assessment process must be an evaluation of the presence of sepsis, neuropathy, biomechanics of the foot and the presence of PAOD (in order to try and define whether there is ischaemia as a consequence of large vessel occlusive disease).

Local vascular assessment includes clinical examination with some form of radiological imaging. However investigative algorithms vary between institutions and in other centres a variety of haemodynamic and/or biological assessments may be incorporated into this process.

1.3.1 Clinical assessment

DFU may be broadly characterized into two groups – neuropathic and neuroischaemic. The neuropathic foot will typically be warm and well perfused, with palpable foot pulses, a reduction in hydrosis may be reported and there can be fissuring of the skin. The neuroischaemic foot will generally appear normal (although in some cases may appear hyperaemic as a consequence of arteriovenous shunting) and may be cool on palpation. There may be DFU. A history of intermittent claudication may be volunteered, which implies clinically significant PAOD, although in patients with DM (who will frequently have a more distal pattern of arterial disease – crural artery involvement [Strandness 1964, Hiatt 2001, Rueda 2008]) claudication may not be present.

Arterial pulse palpation is the central component of clinical assessment of the peripheral circulation of the lower limb. Arterial pulse palpation will include assessment of the femoral pulse (common femoral artery), popliteal pulse (popliteal artery), posterior tibial pulse (posterior tibial artery) and dorsalis pedis pulse (dorsalis pedis artery – continuation of the anterior tibial artery). The presence (or absence) of a pulse gives the clinician an indication about the state of the circulation in the arterial segment above. The sensitivity and reproducibility of proximal pulse palpation (femoral pulse) is 95% [Brearley 1992]. However the accuracy of distal pulse palpation would appear to be poor with under-diagnosis in as many as 30%, false-positives and significant inter-observer variation described [Criqui 1985, Myers 1987, Brearley 1992, Lundin 1999].

Although the 'character' of a pulse is widely described, pulse character is not helpful in the context of assessment of the peripheral circulation. A binary assessment of 'normal' or 'abnormal/absent' is likely to provide more consistent clinical information, with the latter implying a high probability of proximal PAOD [Jacobsen 1990], although clearly the burden of PAOD cannot be described from pulse palpation in isolation.

Given that the clinical course of DFU is in many cases protracted, there may be a role for regular ulcer assessment whilst podiatry and dressing care is provided and more complex investigation is being performed. As a clinical predictor of wound healing, Sheehan has described the utility of serial ulcer measurement over a four-week period of intensive ulcer care [Sheehan 2003]. However there are significant limitations to this clinical assessment,

specifically the positive predictive value of healing was only 58%, whilst the negative predictive value was 91%.

1.3.2 Non-invasive haemodynamic assessment

The cornerstone of non-invasive vascular assessment of the lower limb is the ankle/brachial pressure index (ABPI), the principle for which was described by Windsor in 1950 [Windsor 1950]. For the detection of POAD, defined as the presence of a 50% stenosis or more ABPI has a sensitivity of 79-95% and specificity of 90-96% (in the absence of diabetes) [Carter 1968, Yao 1968, Lijmer 1996, Doobay 2005]. The Society for Vascular Technology has published a comprehensive laboratory protocol for assessment of ABPI, which aims to control the methodology by creating reproducible conditions [Cole 2001].

However there are a number of potential issues that can limit the accuracy of ABPI. If there is significant collateralization of the aorto-iliac segment some patients may have a normal resting ABPI even in the presence of significant proximal stenotic/occlusive atherosclerotic arterial lesions [Elhadd 1999]. Although there are well-defined protocols for assessment, ABPI may be recorded inconsistently through variability in methodology. Inter-observer variability has also been reported [Osmundson 1985, Simon 2000]. Furthermore determination of ABPI in patients with DM and PAOD is recognised as being less reliable than in patients without DM and PAOD [Raines 1976, Formosa 2013]. Pathophysiologically this is attributed to smooth muscle hypertrophy compounded by calcification of the media (there is increased ossification of the arterial wall due to increased expression of bone morphogenetic protein) leading to loss of arterial distensibility and increased vessel stiffness [Townsend 2008]. In the clinical context of minor amputation in patients with DM, ABPI in isolation has been described as a poor indicator for wound healing (or the potential requirement for arterial reconstruction) [Caruana 2015].

Toe pressures (TP) and the calculation of a toe brachial index (TBI) represent another non-invasive means of assessing peripheral arterial pressure. It has been argued that in patients with DM TP/TBI may be more representative of the haemodynamic status of the limb than ABPI as the digital arteries are less likely to be effected by medial calcification. TASC define a TP of less than 30mmHg as indicative of critical limb ischaemia. A number of

studies have described the relationship of TP and wound healing. It has been suggested that no healing of primary forefoot amputations is seen if the TP is less than 38mmHg and that there are no failures of wound healing if the TP was 68mmHg, (or if the TP increased by 30mmHg following revascularisation) [Larsson 1993, Vitti 1994]. However there is limited evidence validating this. Of the published work on TP/TBI in a normal study population only one of eight studies excluded asymptomatic PAOD by performing concurrent arterial imaging. From an epidemiological perspective ABPI is predictive as marker for cardiovascular morbidity and mortality, but with TP/TBI no firm conclusions can be drawn from the current literature [Høyer 2013]. Much of the published work describes assessment using the hallux (and more recently) the second toe [Bhamidipaty 2015]. Whether similar changes are seen in the other toes of the foot is uncertain. Finally although much of the evidence describing TP/TBI seem to be compelling more recent work has questioned the accuracy of the current haemodynamic criteria to reflect patients at higher-risk of major limb amputation [Vallabhaneni 2016] – this may be particularly relevant for patients with DM as the disease process seems to follow a different course from patients with PAOD in isolation [Leese 2007, Elgzyri 2013].

1.3.3 Other tests of peripheral perfusion

Pulse wave velocity evaluates changes in arterial pulse waves and is inversely related to the square root of the compliance of the vessel. Assessment of pulse wave velocity as an investigative strategy in patients with DM was described in 1962 [Woolam 1962]. It is an established technique that can be used to evaluate arterial compliance between two points in the arterial tree. There is some data to suggest that in the context of patients with POAD pulse wave velocity may correlate with ABPI and as such may be a valuable screening tool [Khandanpour 2006]. The investigation may augment haemodynamic assessment with ABPI/TBI and help identify a patient population at higher risk of cardiovascular events [Sutton-Tyrrell 2005]. However there is no high quality contemporary evidence that it has predictive value with regard to DFU healing.

Skin auto fluorescence is another non-invasive investigative tool that can predict vascular insufficiency in patients with type II DM [Lutgers 2006]. The technique of skin auto fluorescence has been linked to the formation of advanced glycosylation end products, which are increased in diabetes [Brownlee 2001] and which have a deleterious effect on the arterial wall. As with pulse wave velocity this investigation can help to identify a

population at risk of cardiovascular events [Meerwaldt 2005], but there is no evidence to support predictive value with regard to DFU healing.

Hyperspectral technology (HT) is a diagnostic tool that can quantify tissue oxygenation and has been shown to detect systemic and local microcirculatory changes associated with diabetes. Khaodhiar *et al* describe HT as an accurate predictor of ulcer healing (although it must be noted that their study included only a small number of patients with Type I DM and patients with POAD were excluded) [Khaodhiar 2007]. There is some conflicting evidence as to the utility of HT in patients with PAOD [Jafari-Saraf L 2010, Chin 2011] and the technique requires further clinical validation before it may be used safely and effectively in a clinical setting.

Transcutaneous oxygen tension measurement ($TcPO_2$) is a non-invasive investigative modality that can be used to assess the partial pressure of oxygen at the surface of the skin. The normal oxygen tension in the foot is 60mmHg. $TcPO_2$ has been investigated in patients with PAOD, with and without DM [Wyss 1984]. In both groups $TcPO_2$ measurements of less than 20mmHg were associated the presence of ulceration and a potential requirement for major limb amputation. However patients with DM were more likely to have ulceration with $TcPO_2$ greater than 20mmHg, confirming (as previously described) the multifactorial nature of DFU.

Subsequent studies with relatively small groups of patients have suggested $TcPO_2$ mapping may have a role in defining the patient population with DFU who have a significant ischaemic component as a consequence of PAOD that could potentially be modified to alter the natural history of the disease process [Ballard 1995] and possibly prediction of DFU ulcer healing [Kalani 1999]. A potential confounding issue is that $TcPO_2$ in patients with DM, but without PAOD is lower than is found in age/sex-matched controls [Meijer 2008] and is consequently this technique is not specific to PAOD.

1.3.4 Radiological imaging

Radiological imaging provides robust anatomical information with regard to the burden, morphology and pattern of PAOD and is essential to plan intervention – it does not allow meaningful assessment of distal perfusion. Interpretation of arterial imaging in the DM cohort is challenging – frequently there is multi-level arterial disease and as has been

previously described there is often an infra-geniculate pattern to this [Strandness 1964, Rueda 2008]. These arteries are of small calibre and the combination of calcification of atherosclerotic plaque with concurrent medial calcification, results in challenges with interpretation.

Duplex ultrasound is a non-invasive, inexpensive strategy that obviates the requirement for potentially nephrotoxic contrast media. Duplex ultrasound can provide accurate anatomical information [Eiberg 2010] particularly in relation to the supra-geniculate circulation. Assessment of the proximal tibial vessels can be more challenging, particularly if there is significant calcification. Sequential stenoses as is often encountered in patients with DM can contribute to making assessment of the more distal circulation challenging. With this imaging modality there is also potential for inter-observer variability [Ubbink 2001].

The evolution and progression of Computed Tomography angiography (CTA) with multi-detector spiral scanners has made high quality angiography possible. As the technology has evolved acquisition times have shortened and with improvements in resolution and the use of advanced post-processing techniques image quality has improved. The technique requires the administration of iodinated contrast and consequently there is a risk of contrast-induced nephropathy, which appears to be most significant in patients with a diagnosis of DM and diabetic nephropathy, with pre-existing biochemical renal dysfunction [Parfrey 1989, Waybill 2001]. With this imaging modality sensitivity and specificity appear to be better for arterial occlusions than stenoses, with little inter-observer variability for lesions in the aorto-iliac and femoro-popliteal segments. As with duplex ultrasound assessment of the crural circulation can be more challenging. As previously described these are small calibre arteries and the combination of this with medial calcification and calcification of atherosclerotic arterial plaques can make assessment of vessel patency challenging [Fleischmann 2006].

Magnetic resonance angiography (MRA) can provide high quality arterial imaging using either a contrast enhanced approach or by using non-contrast enhanced, flow sensitive techniques, so called ‘time of flight’, that is effective by measuring the different signal properties between static tissue and flowing blood. The high quality images that can be derived must be balanced against the expense of the investigation, which is time consuming and which many patients find claustrophobic. Again there can be issues with

tibial vessel resolution – there is a shorter time difference between the arterial and venous enhancement, which can result in significant venous contamination [Pollak 2012].

Digital subtraction angiography dates back to the 1970's and the evolution of this technique has been credited to the department of clinical radiology in the Cleveland clinic. The technique is invasive (requiring percutaneous arterial access) and a contrast load must be given (this can take the form of either iodinated contrast – where there is the potential for renal dysfunction as has been described above, alternatively carbon dioxide may be used). The investigation also requires exposure to radiation (to both the patient and the clinical team involved). Many still consider this to be the 'gold-standard' arterial investigation [Pomposelli 2010], as it has both diagnostic utility and offers the potential to perform endovascular intervention (angioplasty and/or stenting). Imaging of the crural circulation and pedal arteries with this investigation is excellent.

The Angiosome concept merits consideration at this stage. The ankle and foot may be sub-divided into six distinct geographical territories, supplied by the posterior tibial, peroneal and anterior tibial arteries. The concept has attracted interest particularly in patients with DFU. There is some evidence in the published literature supporting a strategy of direct revascularisation of the source artery whenever appropriate, rather than relying on collateralization [Attinger 2006, Alexandrescu 2008, Neville 2009]. However the general quality of the evidence available is poor. Published data has generally been derived from single institutions and has been analysed retrospectively. To date there are no prospectively collected data available to validate the reported series.

1.4 Biomarkers in cardiovascular disease

A biomarker may be defined as a measurable indicator of the presence or severity of a disease process. To be effective a biomarker must be both sensitive and specific to the disease process being investigated. It should be easy to measure and the result should offer a meaningful impact upon the management of the condition. Ideally the test should also be inexpensive. The best example of an effective biomarker in current practice is serum troponin assay for the detection of myocardial ischaemia. The investigation has been widely adopted in contemporary clinical practice as it can be measured rapidly and provides useful prognostic information with early identification of patients at higher risk of cardiac death [Antman 1996].

A series of biochemical markers have been identified which reflect endothelial dysfunction and appear to be influenced in the context of both the ischaemic and neuropathic diabetic limb. These include von Willebrand Factor (vWF), Vascular Endothelial Growth Factor (VEGF), Homocysteine, Endothelin-1, Vascular Adhesion Molecule, Thrombomodulin and E-selectin. It is hypothesised that the production of these is potentiated by oxidative stress in the context of white cell activation and reactive oxygen species [Hickman 1994, Woodman 2002]. Hyperinsulinaemia and hyperglycaemia appear to have no apparent effect on release of these biochemical markers [Kessler 2001, Oomen 2002].

vWF is an acute phase protein. Plasma vWF is proposed to be a marker of endothelial dysfunction as it is predominantly synthesised by endothelial cells, which normally account for more than 85% of vWF in circulating blood, along with equimolar amounts of pro-peptide (vWF:AgII) [Vischer 1998]. There is some evidence to support the hypothesis that vWF is a marker of platelet activation, as in pathological conditions it may also be released by platelets [Mannuci 1998]. Plasma vWF is increased in patients with lower limb ischaemia, but is also elevated in patients with DM and neuropathic lower limb ulceration, relative to case matched controls. This appears to be independent of PAOD [Blann 1995] and may rather represent endothelial dysfunction, previously referred to as 'microvascular' or 'small vessel disease'.

Nitric oxide (NO) is a free radical synthesised from L-Arginine by the enzyme nitric oxide synthase. It is a vasodilator secreted by endothelium. As the half-life is short, indirect measures of NO are made by assessment of plasma or urinary nitrates/nitrites. Insulin stimulates NO production through the endothelial insulin receptor, with lack of insulin, or insulin resistance potentially depressing NO production [Zeng 1996]. In wounds NO is produced by macrophages, fibroblasts and endothelium. Jude et al have made several interesting observations relating to the presence of increased NO in diabetic patients with ulceration/healed ulceration relative to diabetic patients without ulceration [Jude 1999]. They also postulate that NO may be involved in the progression of DFU [Jude 2001].

Tissue factor (TF) is a key initiator in the coagulation cascade and has a critical role in the propagation of thrombus. Elevated levels of TF have been demonstrated in patients with DM [Zumbach 1997] and it is postulated that a hypercoaguable state may be associated with adverse outcomes [Soejima 1999].

C-reactive protein (CRP) is a pentameric protein synthesised in the liver in response to interleukin-6 (IL-6), but is also synthesised in smooth muscle cells of atherosclerotic plaques [Khawaja 2009]. CRP is associated with down regulation of NO synthase, increased expression of Cellular Adhesion Molecules (CAMs), activation of the compliment system, up regulation of LDL phagocytosis by macrophages and vascular smooth muscle migration, proliferation and neo-intimal formation. CRP is a marker of vascular inflammation and plaque instability [Khawaja 2009], and several studies including the Rotterdam [Van der Meer 2002] and Edinburgh Artery study [Tzoulaki 2002] have implicated an elevated CRP in the progression of arterial disease.

Interleukin-6 (IL-6) is an inflammatory cytokine, which is produced by hepatocytes, lymphocytes and endothelial cells. As previously described it is the stimulus for the production of CRP and in the Edinburgh Artery Study was found to be the strongest biochemical indicator of progressive arterial disease [Tzoulaki 2005].

It is not clear whether these biochemical markers can meaningfully help identify the patient population with DM at greatest risk of complication, or those that may benefit from revascularisation. There seems to be an association between insulin resistance and endothelial dysfunction and it may be that an increase in endothelial markers such as endothelin-1 and vWF may precede the development of ulceration [Stehower 1996, Seligman 2000]. In a series of patients with type II DM, Lim et al demonstrated a significant reduction in vWF with multi-factorial pharmacological intervention in patients with no pre-existing cardiovascular disease [Lim 2004]. Similarly it would appear that major limb amputation in the context of critical limb ischaemia leads to reduction in vWF as well as VEGF [McLaren 2002, Newton 2008]. It is not clear whether these changes may be seen in patients undergoing revascularisation procedures. Tsai et al describe the effect of lower limb bypass in 21 patients with critical limb ischaemia and type II DM on plasma concentration of endothelin-1 and nitric oxide and found no significant differences pre- and post-revascularisation surgery [Tsai 2009]. It seems unlikely that these markers have the specificity or sensitivity to be useful in a prediction model for patients with DFU. These changes may be useful for monitoring or biochemically assessing the impact of an intervention, but they will not provide any prognostic information with regard to the outcome of DFU and as such it would seem that at best they have limited clinical utility in this setting.

Fundamentally CLI as a consequence of PAOD is a disease of the skin of the foot [Rossi 2004]. As such it would seem that the most appropriate place to identify a biomarker of ischaemia in the context of DFU is the skin of the foot.

1.5 Connexins

1.5.1 Connexin Biochemistry and the Gap Junction

Gap Junctions (GJ) directly link the cytoplasm of adjacent cells. They facilitate inter-cellular communication and are integral in the maintenance of cellular homeostasis. Since the discovery of GJ numerous studies of structure and function have been performed [Caspar 1977, Makowski 1977, Makowski 1982, Maeda 2011]. It has become clear that GJ are integral in the control of cellular differentiation and growth, whilst also representing an important mechanism for apoptosis and spreading death signals between cells (Talhouk 2008, Bargiotas 2009).

The single transmembrane protein subunits of GJ are the Connexin proteins (Cx). Twenty-one different human Cx have been identified each with a unique amino-acid sequence and specific biochemical properties. The genes encoding for Cx tend to occur in clusters around certain chromosomes, sharing a common structure, consisting of two exons separated by one intron sequence.

The Cx protein is composed of nine principle domains. The N-terminus, two extracellular loops (EL1 and EL2), stabilized by intra-molecular disulfide bridges, and four transmembrane domains, which are highly conserved among different isoforms. The intra-cellular amino acid terminus is of similar length in all Cx. The major difference between Cx is the length and sequence motifs of the carboxyl tail, which are divergent allowing for functional differences between the different Cx and the connexon types [Evans 2002, Dbouk 2009]. The precise purpose of multisite phosphorylation of the carboxyl tail is unclear. They access the cytoplasmic reticulum and bind other structural proteins, a process which is crucial for synchronized cellular communication.

Cx nomenclature is based on molecular weight; for example, Cx43 is a 43 kiloDaltons (kDa) protein [Beyer 1987]. A second method of nomenclature is based on the sequence

similarity and length of the cytoplasmic loop (CL). This latter methodology separates Cx into four main groups (alpha, beta, delta and gamma) [Eiberger 2001].

Regulation of Cx expression is effected at different levels from transcription to the post-translational processes although the mechanisms involved are poorly defined. It has been suggested that microRNA (mRNA) species may be key regulators of Cx expression. Cx are synthesised in the endoplasmic reticulum, where they are rapidly oligomerised to form hexameric connexons (CxHc). This process is completed within the Golgi apparatus, following which the connexon is trafficked to assemble in the lipid milieu of the plasma membrane (a calmodulin dependent step). The polymeric assemblies of aligned pairs results in the formation of GJ.

Cx proteins have a very short half-life not exceeding few hours [Solan 2009], the synthesis and delivery to the membrane of new Cx proteins is coupled to simultaneous gap junction internalization and Cx degradation [Segretain 2004]. The entire GJ or fragments of the GJ internalise as double membrane structures, with their final fate being a merger with lysosomes where they are degraded – breakdown of channels occurs via endocytosis and degradation [Salameh 2006].

CxHc are arranged into dynamic, spatially and temporally organised aggregates, which at the ultra-structural level are identified by their electron dense pentalaminar structure [Hanner 2010]. These are often described as plaques [Segretain 2004], with a single plaque containing anything from a few, too many thousands of individual channels. These plaques can facilitate the rapid propagation of action potentials and the direct diffusion of inorganic ions, ATP, cAMP, and other small water-soluble molecules (<1.0-2.0 kDa) between cells.

The structure of the GJ channel pore is critical to understanding the molecular mechanisms underlying selective permeation and voltage-dependent gating of channels formed by the Cx family [Tang 2009]. The capacity of different Cx isoforms to respond uniquely to similar stimuli and thereby alter the selectivity and permeability properties of GJ is a key concept in understanding Cx-specific functional roles and an important reason for the large number of Cx isoforms and their co-expression [Harris 2007, Heyman 2009].

Cx expression, hemichannel function and GJ channel activity are closely regulated. This is mediated by growth factors and pro-inflammatory mediators such as peptidoglycan (PGN),

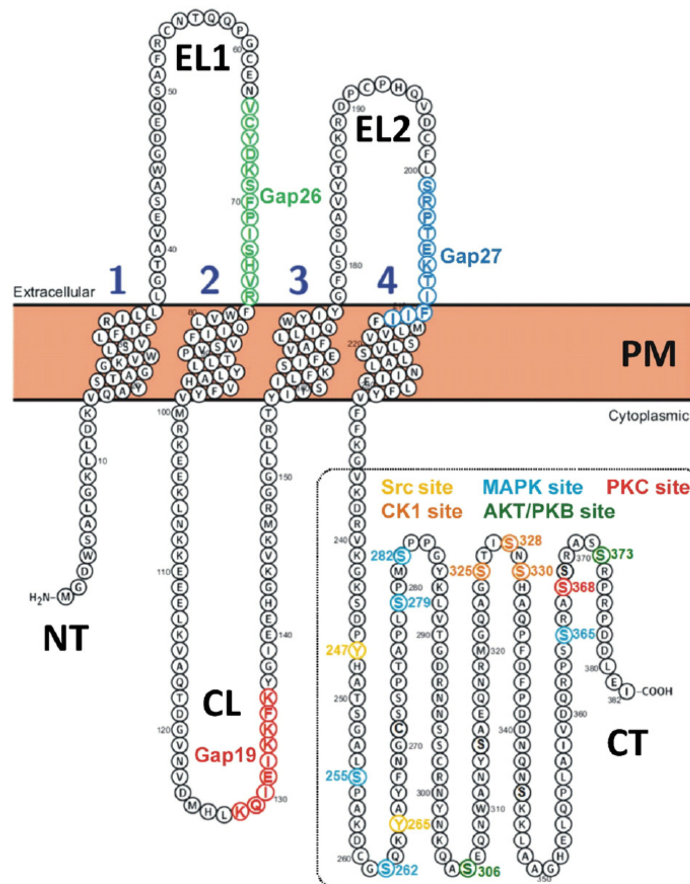
lipopolysaccharide (LPS) and the cytokines [Chanson 2005]. Cytokines may have a role in gene expression, stability of Cx mRNA and protein degradation. Although pro-inflammatory cytokines exacerbate cellular damage, they also have an important role in tissue repair by stimulating apoptosis and cell proliferation. At a molecular level the effects of these substances is termed 'channel gating' [Anand 2005, Chanson 2005]. The term gating has been defined as the mechanism by which the movement of ionic or non-ionic species becomes physically restricted because of the channel itself. Gating may also induce a conformational change of a channel protein, a reversible process where a complete or relative closure or opening of a channel occurs [Moreno 2005, Moreno 2007]. The domains involved in Cx channel gating include the carboxyl and amino-termini, as well as the extracellular domains. Inducers include electrical current and chemical changes (pH, protein phosphorylation and intra/extracellular Calcium). The diverse actions of phosphorylation may induce a variety of modulation and gating properties in cells.

Under normal conditions GJ channels are open. Conversely CxHc generally remain in a closed configuration but may be induced to open by a variety of stimuli (such as removal of extracellular calcium, hypoxia, mechanical stress and dephosphorylation), with a subsequent release of ATP, which may then mediate autocrine or paracrine signaling via purinergic receptors [Bader 2006]. CxHc have a functional capacity out-with GJ formation, as they represent large unopposed membrane pores that connect the intra and extra-cellular compartments. These hemichannels can be induced or opened by elimination of extracellular calcium or inhibition of the electron transport chain. Of potential pharmacological relevance is that CxHc may be blocked by the peptide gap junctional inhibitors [Figeuroa 2006].

1.5.2 Connexin 43

Cx43 is broadly expressed and is the most abundant of the Cx being present in in at least 34 tissues and 46 cell types [Zimmerli 2007, Wright 2009, Solan 2009]. There are sixteen sites on the carboxyl terminus of Cx43 that may be phosphorylated. Figure 1.3 provides a schematic representation of Cx43 [Schulz 2015].

Figure 1.3: Connexin 43 [Schulz 2015]



In cultured fibroblasts initial phosphorylation of Cx43 may occur within 15 minutes. A basal degree of phosphorylation seems to be required on five of the serine residues of Cx43 for the assembly and functioning of GJ. Most Cx are phosphorylated in vivo, primarily on the serine and to a lesser extent on the threonine and tyrosine residues of the carboxyl terminus [De Vuyst 2007].

Serine368 is a major site of phosphorylation on Cx43 in vivo. PKC mediated phosphorylation of the Serine368 of Cx43 [Cx43(Ser368)] is a major mechanism by which TPA disrupts cellular communication (Lampe 2000). Activation of several kinases including protein kinase A (PKA), protein kinase C (PKC), p34(cdc2)/cyclin B kinase, casein kinase 1 (CK1), mitogen-activated protein kinase (MAPK) and pp60(src) kinase can lead to phosphorylation of the majority of the 21 serine and two of the tyrosine residues in the C-terminal region of Cx43 [Solan 2005].

1.5.3 Connexin/Gap Junction Expression in Skin and Wound Healing

Growth control and maintenance of a homeostatic equilibrium in the mature mammalian epidermis is manifested morphologically by a constant thickness of epidermal cell layers. Cells within the basal layer of the skin represent the proliferative population, supplying cells that move into the supra-basal layers and undergo a specific and coordinated program of differentiation (including the expression of the substrates involucrin and loricrin and the cross linking enzyme transglutaminase).

The epidermis is primarily composed of keratinocytes that temporally and spatially express up to 10 different Cx types depending on the degree of differentiation.
Cx43 is ubiquitously found in all epithelial layers of ectodermal origin [Richards 2005]. There is focal distribution of Cx43 expression in the basal keratinocytes (stratum basale), with the suprabasal cells (stratum spinosum) demonstrating higher expression [Richard 2000, Brandner 2004]. Cx43 expression in normal proliferating keratinocytes falls both at the mRNA and protein level in non-dividing cells (post-confluent cells) [Gibson 1997].

Butterweck and co-workers have used a murine model to investigate epidermal and follicular development and differentiation, demonstrating that the development of healthy skin coincides with multiple differential Cx expression [Butterweck 1994]. The co-expression of multiple different Cx from the same keratinocyte, with co-localisation at GJ suggests that the epidermis can form communication compartments with different regulatory properties [Risek 1998]. Direct intercellular signaling, controls keratinocyte behaviour, proliferation and differentiation, occurs through gap junctions.

The GJ of human keratinocytes predominantly express Cx43, which is abundantly expressed within the interfollicular epidermis and is involved in the complex process of keratinocyte differentiation [Saloman 1994, Gibson 1997, Wiszniewski 2000]. In the undifferentiated epidermis Cx26 and Cx43 are co-expressed and differentially regulated during the differentiation of tissue. Previous studies have not demonstrated the presence of Cx26 in the fully differentiated epidermis [Saloman 1994]. The maintenance of an established epidermis does not seem to be affected by Cx availability.

Wound healing is a complex process involving four main phases - haemostasis,

inflammation, proliferation and remodeling [Chin 2011]. The inflammatory cascade initiated results in a variety of cells migrating into the wound (including the neutrophil, which releases oxygen free radicals and secretes pro-inflammatory cytokines and macrophages, which are major producers of cytokines, chemokines and growth factors that direct subsequent cell migration). Thereafter, subsequent remodeling and scar differentiation involves multiple cell types in a predictable and overlapping sequence of cellular events that includes inflammation, migration/proliferation and extracellular matrix deposition [Rhett 2008].

GJ expression is altered at sites of inflammation in vivo and could thus contribute to pathophysiological responses. Cx expression is tightly regulated after skin injury, at the transcriptional, translational, and post- translational levels. Cx43 would seem to be central to this process, with Langlois and colleagues describing dysregulation of keratinocyte differentiation resulting from changes in Cx expression (Langlois 2007). Prior to GJ formation, under specific physiological conditions Cx may be induced to open and release paracrine messengers into the extracellular space. In this context Cx have a role in the proliferation, migration and differentiation of keratinocytes and fibroblasts. Subsequent signaling via the extensive GJ network found in skin [Goliger 1995] and from within communication compartments of the advancing epithelium is proposed to play a central role in the repair process [Wang 2007]. The extensive interaction between keratinocytes and fibroblasts during this stage of the healing process is fundamental and correlates with changes in Cx expression [Abdullah 1999].

Epidermal cells at the wound edge undergo several phenotypic alterations called keratinocyte activation to permit migration across the wound. Approximately 24-48 hours after injury cells at the wound edge become hyper-proliferative and generate additional migratory cells [Goliger 1995]. Following injury, spontaneous epidermal regeneration occurs over a five to seven day period. A role for GJIC in the wound healing process was postulated by Gabbiani et al, who demonstrated with electron microscopy, that epithelial cells at the periphery of a healing wound have nearly four times the surface area occupied by GJ than normal cells [Goliger 1995]. This is perhaps unsurprising given that Cx have been implicated in a variety of different dermatological diseases (keratitis-ichthyosis deafness syndrome, erythrokeratoderma variabilis, Vohwinkel's syndrome).

Down-regulation of Cx43 in the basal layer cells of the epidermis may favour the transformation or de-differentiation of keratinocytes into the migrating phenotype [Kretz 2003]. In the proliferative phase expression and phosphorylation of Cx43 at the wound edge then increases [Chin 2011], with an increase in the expression and phosphorylation status at Cx43(Ser368) in adjacent basal cells [Solan 2009]. By seventy-two hours the levels and distribution of Cx43(Ser368) have returned to normal.

1.5.4 Influence of diabetes on Connexin expression in skin and wound healing

"Hyperglycaemic memory" is a phenomenon where, even after glycaemic control has been re-established in a diabetic patient, the effects of the prior hyperglycaemia insult continue to develop. The onset of diabetes has a marked effect on the expression levels of Cxs in the skin, an effect that is more pronounced when the skin is injured. The mechanism whereby diabetes alters the expression of Cxs, with a differential effect on different Cx proteins has not been fully explained.

Both high glucose and oxidative stress *in-vitro* have been shown to alter Cx expression and/or gap junctional communication in a variety of different cell types [Wang 2007, Ball 2011, Pollock 2011]. In the hyperglycaemic state mRNA transcription demonstrates increased expression of Cx26, Cx30.3, Cx31.1 and Cx43 [Bajpai 2011]. The hyperglycaemic state influences PKC activation and through this metabolic pathway may have different effects on Cx43 [Malfait 2001].

In diabetes, impaired wound healing and ulceration, by glycation of extracellular matrix compounds, inhibit cellular migration and increase sensitivity of fibroblasts to growth factors [Pollock 2011], with delayed entry into the wound site and a reduction in collagen production [Abdullah 1999]. Hyperglycaemia has a directly detrimental affect on the migration of epidermal keratinocytes and dermal fibroblasts, with evidence from in vitro models suggesting that this may be the result of down regulation of Cx43 expression, although this does not appear to be uniform and may be a direct consequence of the diabetic condition rather than a secondary consequence, or complication of long-term diabetes. There is some evidence that this inhibition may be due to an increase in protein kinase C phosphorylation [Wang 2007, Tyml 2011].

In healthy skin Brandner and co-workers have evaluated the keratinocytes in human subjects with and without diabetes. The biopsies were taken from a proximal site (from a level at which there was unlikely to be arterial insufficiency). The expression of Cx26 was no different between individuals with and without diabetes (skin biopsy) and there was no statistical difference in the expression of Cx43 mRNA between DM and non-DM subjects [Brandner 2008].

Persistent abnormal up-regulation of Cx43 in DM has been demonstrated in animal models [Wang 2007]. This has also been demonstrated in chronic non-healing wounds and in the epidermal wound edges of chronic diabetic ulcers in humans [Wright 2009]. Following injury the Cx43 levels in diabetic skin increase rather than down-regulate (down-regulation in diabetes seems to be delayed by at least forty-eight hours). The keratinocytes in diabetes seem to form a 'thick bulb' with a delay in adopting a migratory morphology [Chin 2011]. The behaviour of fibroblasts and expression of Cx43 and Cx26 in DM and non-DM skin seems to be similar [Abdullah 1999].

1.5.5 Connexin 43 and ischaemia

Ischaemia may be defined as a complex insult comprising of hypoxia, acidosis, local and systemic metabolic changes and leading to ATP depletion at a cellular level. The effect of ischaemia on Cx expression has been investigated in the cardiac myocyte and myofibroblast, but has not been fully defined in skin. There is evidence to suggest differences in Cx43 expression in response to ischaemia. Prolonged hypoxia in cardiac models down-regulates Cx43 GJs (reduction of 50% at 5 hours) and there is a total reduction of cellular Cx43 [Zeevi-Levin 2005]. However there appears to be a rapid increase in phosphorylation of Cx43(Ser368) [Solan 2007]. From a functional perspective ischaemic conditions influence both hemichannel and GJ function (for example metabolic inhibition may open Cx43 hemichannels, where-as ischaemia may induce GJ closure) [Johansen 2011].

Consequently in the cardiac myocyte hemichannel opening in response to ischaemia may be a factor in the development of cell death through alteration in membrane properties as a result of ionic dysregulation (calcium loss), loss of metabolites and changes in intracellular ATP [Shintani-Ashida 2007, Talhouk 2008, Bargiotas 2009]. The phosphorylation status of the carboxyl tail amongst other factors may regulate this. Cx43(Ser368) is a major

protein kinase C phosphorylation site. In the cardiac myocyte it seems to be involved in ischaemic pre-conditioning, by delaying dephosphorylation (Johansen 2011). It is not clear what role this may have in human skin.

1.5.6 Connexin 43(Serine 368) as a biological marker of ischaemia in skin

Previous work has demonstrated the presence of Cx43(Ser368) in healthy skin of the lower leg – with significant up regulation of Cx43(Ser368) 24 hours following mechanical wounding (punch biopsy) [Richards 2004]. Cardiac myocyte work implies down regulation of Cx43 and up regulation of Cx43(Ser368) in response to ischaemia in the cardiac myocyte [Johansen 2011].

Consequently the hypothesis for this study was that Cx43 and Cx43(Ser368) protein expression would be modified in response to ischaemia in human skin

1.6 Study Aims

i. To determine whether UoT classification on the first presentation for a particular limb, carried clear prognostic significance in terms of major adverse clinical events, namely hospital admissions for sepsis, the need for arterial reconstruction or amputation (minor and major), ulcer recurrence and death.

ii. To investigate in vivo the expression of Cx43 and Cx43(Ser368) in full thickness skin biopsies from patients with severely symptomatic PAOD (with and without DM) and in a control group

iii. To establish in vitro how protein expression of Cx43 and Cx43(Ser368) in human keratinocytes and fibroblasts (cultured from patients with PAOD, with and without DM and a control population without occlusive arterial disease) is influenced by hypoxia

Chapter 2 – The University of Texas Classification of Diabetic Foot Ulceration

2.1 Description of the University of Texas classification, the value of scoring systems in diabetic foot ulceration and aims of the present work

The process of attaining and maintaining a healed state in patients with DFU remains incompletely understood, but a multidisciplinary approach is clearly beneficial [NICE 2015]. Early identification of patients with poorer prognoses and at higher risk of major adverse clinical events may be helpful. A number of DFU scoring systems have been developed for this purpose [Jeffcoate 1993, Lavery 1996, Mills 2014]. As well as having prognostic significance, scoring systems aid inter-disciplinary communication. Regardless of the scoring system used, simplicity and reproducibility are essential [Teasdale 1974].

The University of Texas (UoT) diabetic foot score has two components, the first describing ulcer depth and the second the presence of sepsis and/or peripheral arterial occlusive disease (PAOD), defined on clinical grounds. The latter are potentially correctable, and correction may influence the natural history of the individual ulcer and the outcome for the affected limb. The UoT scoring system has been retrospectively validated and a significant association observed between ulcer depth and the presence of infection and/or ischaemia with major limb amputation [Armstrong 1998].

Surgeons often become involved in the care of DFU, but frequently this is only after major complications have developed. The UoT classification applied at first presentation may have a role in alerting the multidisciplinary team to limbs at greatest risk, not just of amputation, but also of recurrent ulceration and infection. Targeting these limbs with early investigation and more aggressive management of ischaemia and sepsis may improve long-term outcomes. Consistency of DFU classification with UoT would improve communication between the different members of the multidisciplinary healthcare team.

The aim of this chapter was to determine whether UoT classification on the first presentation, carried clear prognostic significance in terms of major adverse clinical events, namely hospital admissions for sepsis, the need for arterial reconstruction or amputation, ulcer recurrence and death.

2.2 Summary of patients and methods

The South Glasgow diabetic foot clinic database was established in 2005. Data were entered at first presentation and all subsequent clinic attendances. Recorded data included patient demographics, estimated date of onset of the ulcer(s), site of ulceration and classification according to the UoT scoring system. The data for patients entered between May 2005 and August 2013 were used. Median patient follow-up was 3.5 years. Follow-up data were retrieved from the primary database and supplemented using local and national electronic patient record systems through case-linkage using the Community Health Index (CHI) number [Stuart 2013].

All patients presenting to the diabetic foot clinic with DFU were considered eligible for inclusion. For this analysis, the UoT classification at the first presentation to the diabetic foot clinic was used. If there was more than one ulcer at presentation, the highest scoring ulcer was used to classify the individual limb. Patients attending the clinic with Charcot change without concurrent ulceration were excluded from analysis.

For UoT classification, ulcer depth was ascertained by probing, ischaemia diagnosed clinically based on the absence of foot pulses and sepsis defined using previously validated clinical criteria (more than one sign of local inflammation or the presence of purulent discharge) [Lavery 2007]. Assessment was performed and recorded by different members of the multidisciplinary team; including diabetologists, podiatrists, specialist nurses and vascular surgeons.

Major adverse clinical events (MACE) were defined as hospital admissions for sepsis (both medical management and surgical drainage), arterial reconstruction (endovascular or surgical), lower extremity amputation (defined as any loss of the limb in the transverse plane; infra-malleolar procedures were defined as minor amputations and supra-malleolar amputations as major amputations – [Kennon 2012]) recurrent ulceration and death during the follow-up period.

Data were analysed by limb. Summary data are expressed as mean and standard deviations (SD) or median and interquartile range (IQR). Statistical analysis was performed using SPSS (version 21). Comparison of groups was performed with Chi-square test (a P-value

of less than 0.05 was considered significant). Survival analysis was performed using Kaplan-Meier methodology, with the Chi-square test used to compare the curves.

2.3 Results

Details of 388 patients with 515 symptomatic limbs were entered into the database during the study period. Eight patients were excluded from analysis as they presented with Charcot change without active ulceration. From the remaining 507 limbs there were 971 ulcer episodes during the study period.

Median patient age was 66-years (inter-quartile range 54 to 77-years) and 230 patients (59.3%) were men. In 130 (25.2%) cases the patient had multiple ulcers on the index foot at the time of presentation. In 200 cases (38.8%) ulcers occurred at more than one anatomical site on the same foot during follow-up. At presentation 52 (13.4%) patients had bilateral foot ulceration. By the end of the follow-up period 125 (32.2%) patients had developed ulceration affecting both feet.

In 432 (85%) cases the ulcer was defined as superficial and in 230 (45%) cases neither sepsis nor ischaemia were present at the time of the first assessment (Table 2.1). There were only 12 cases where re-assessment of the initial DFU resulted in the UoT score being changed to indicate more severe disease.

Table 2.1 University of Texas classification of index ulcer on primary presentation

	Depth 0 (Epithelialised) n=63	Depth 1 (Superficial) n=369	Depth 2 (Tendon/joint capsule) n=21	Depth 3 (Bone/joint) n=54
Stage A (No sepsis/Pulses present) n=230	55	170	1	4
Stage B (Sepsis/Pulses present) n=75	1	50	8	16
Stage C (No sepsis/Pulses absent) n=148	7	119	11	11
Stage D (Sepsis/Pulses absent) n=54	0	30	1	23

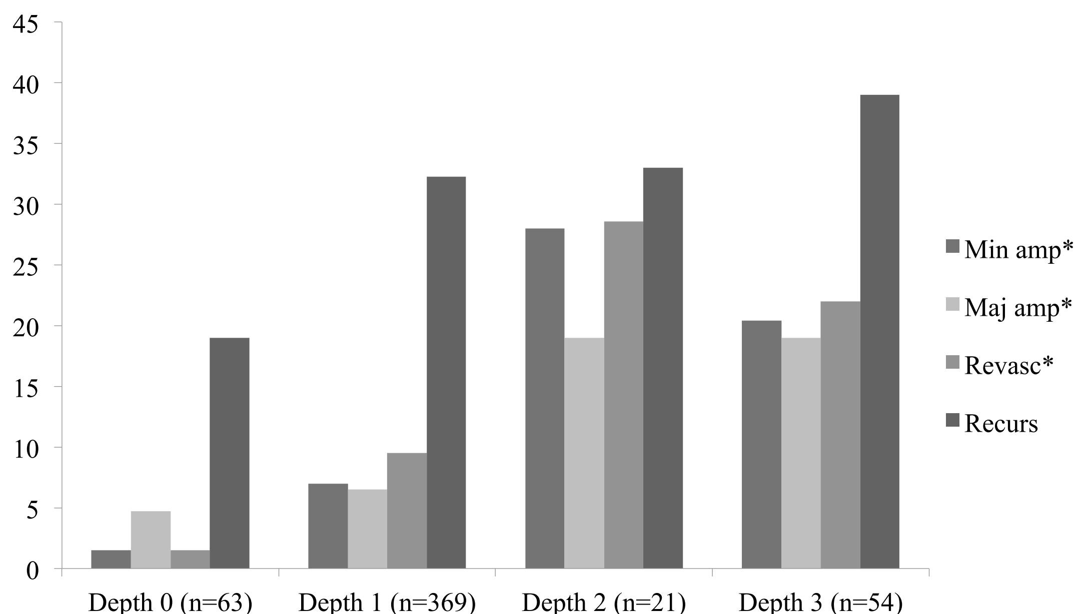
Major adverse clinical events are summarised in Table 2.2. Deeper ulcers at presentation were associated with significantly higher rates of amputation (both minor and major) and arterial reconstruction (Figure 2.1). With progression in the stage of UoT score there was a significant increase in both rates of major limb amputation and arterial reconstruction (Figure 2.2).

Table 2.2 Summary of Major Adverse Clinical Events

	Minor amputation	Major amputation	Revasc*	Recurrence	Sepsis	Death	MACE
0A (55)	1 (2%)	1 (2%)	1 (2%)	10 (18%)	7 (13%)	9 (16%)	15 (27%)
0B (1)	0	1	0	0	1	0	1
0C (7)	0	1	0	1	3	0	3
0D (0)	-	-	-	-	-	-	-
1A (167)	12 (7%)	8 (5%)	6 (3.5%)	33 (20%)	40 (24%)	39 (23%)	74 (44%)
1B (52)	4 (8%)	2 (4%)	3 (6%)	12 (23%)	15 (29%)	6 (12%)	25 (48%)
1C (118)	7 (6%)	8 (7%)	16 (14%)	19 (16%)	32 (27%)	47 (40%)	57 (48%)
1D (30)	2 (7%)	6 (20%)	10 (33%)	17 (57%)	13 (43%)	9 (30%)	16 (53%)
2A (1)	0	0	0	0	1	0	1
2B (8)	3	0	0	3	3	1	6
2C (11)	3 (27%)	3 (27%)	6 (55%)	6 (55%)	4 (36%)	6 (55%)	8 (73%)
2D (1)	0	1	0	0	1	0	1
3A (4)	1	0	0	1	2	1	4
3B (16)	3 (19%)	2 (12%)	0	4 (25%)	8 (50%)	4 (25%)	13 (81%)
3C (11)	1 (10%)	3 (27%)	9 (81%)	0	4 (36%)	2 (18%)	9 (81%)
3D (23)	6 (26%)	5 (22%)	3 (13%)	5 (22%)	13 (57%)	8 (35%)	21 (91%)
Total (507)	43 (8%)	40 (8%)	54 (11%)	111 (22%)	144 (28%)	132 (26%)	254 (50%)

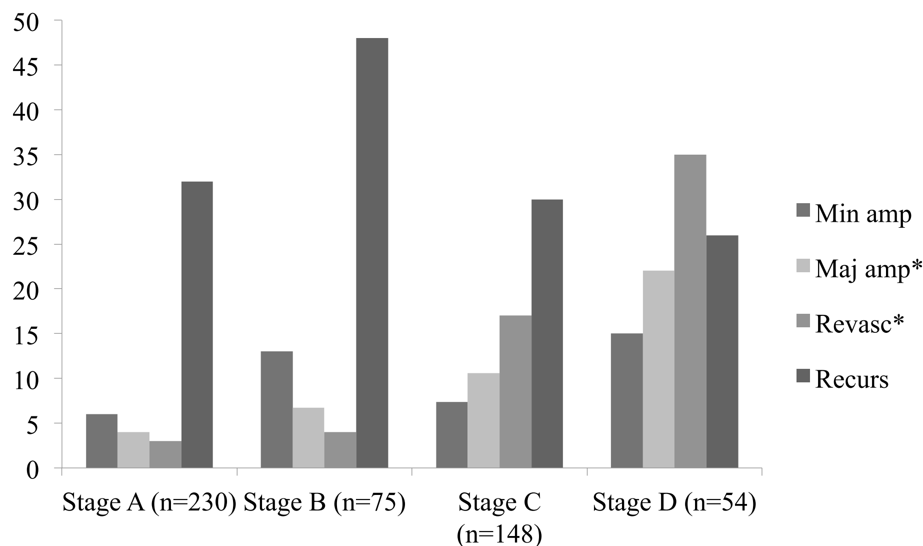
* Includes both endovascular and surgical revascularisation

Figure 2.1 Ulcer depth and major adverse clinical events



*p <0.001

Figure 2.2 Ulcer stage and major adverse clinical events



*p <0.001

There were 144 hospital admissions with sepsis during the follow-up period (the median duration of hospital admission 14 days IQR 6 to 60 days). There were no significant differences between the depth and stage of the ulcer at presentation and a requirement for drainage of sepsis. Increasing ulcer depth and stage were independently associated with

more frequent hospital admissions for sepsis (Figures 2.3 and 2.4) and a greater duration of inpatient stay (Tables 2.3 and 2.4).

Figure 2.3 Septic admissions and management by ulcer depth (p>0.05)

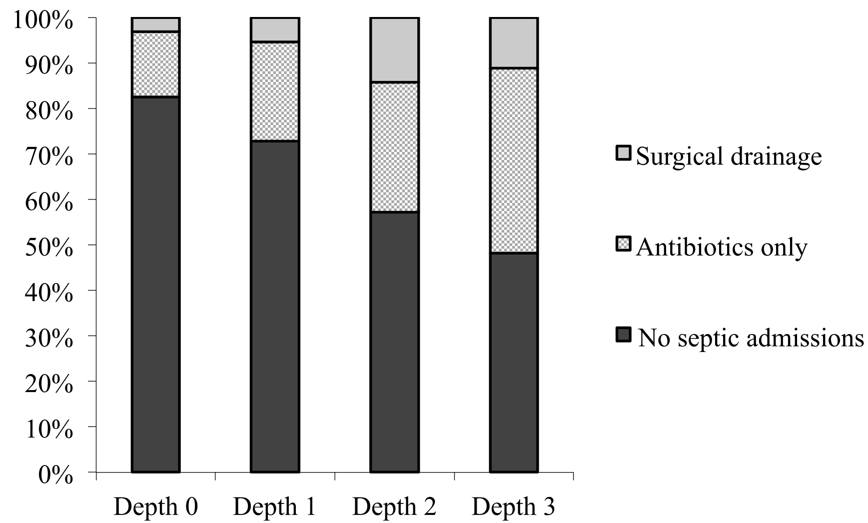


Figure 2.4 Septic admissions and management by ulcer stage (p>0.05)

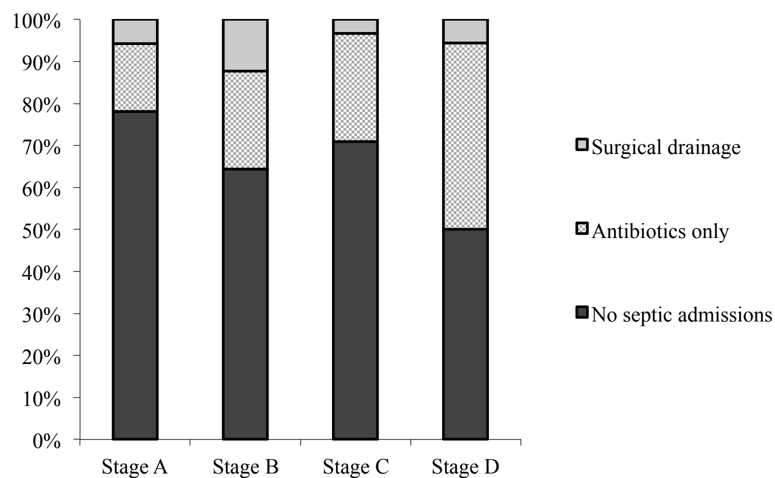


Table 2.3: Inpatient stay by ulcer grade

	Number of Limbs	Mean Inpatient Stay (Days)
Grade A	229	3.9
Grade B	74	9
Grade C	147	5.6
Grade D	54	15.2

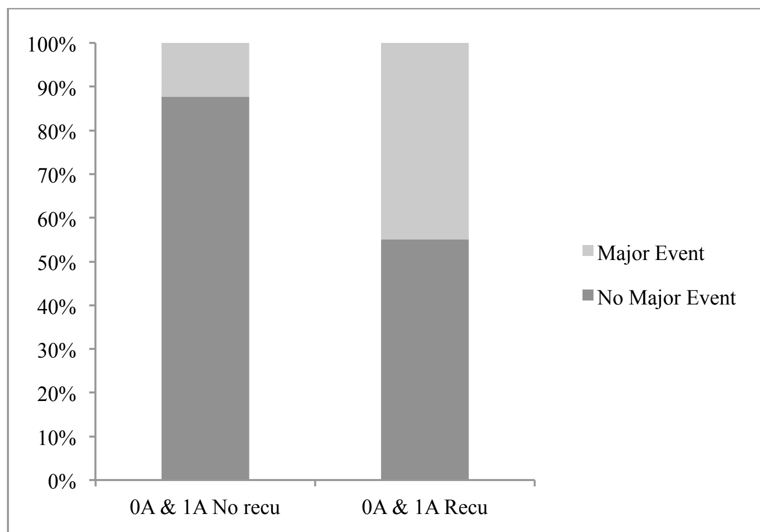
Table 2.4: Inpatient stay by ulcer depth

	Number of Limbs	Mean Inpatient Stay (Days)
Depth 0	63	2.3
Depth 1	366	5.4
Depth 2	21	13.4
Depth 3	54	15.1

The mean time to ulcer resolution was 4 months (range 1 to 63 months) – in 61 (12%) cases there was persistent ulceration despite intervention at the end of the study period.

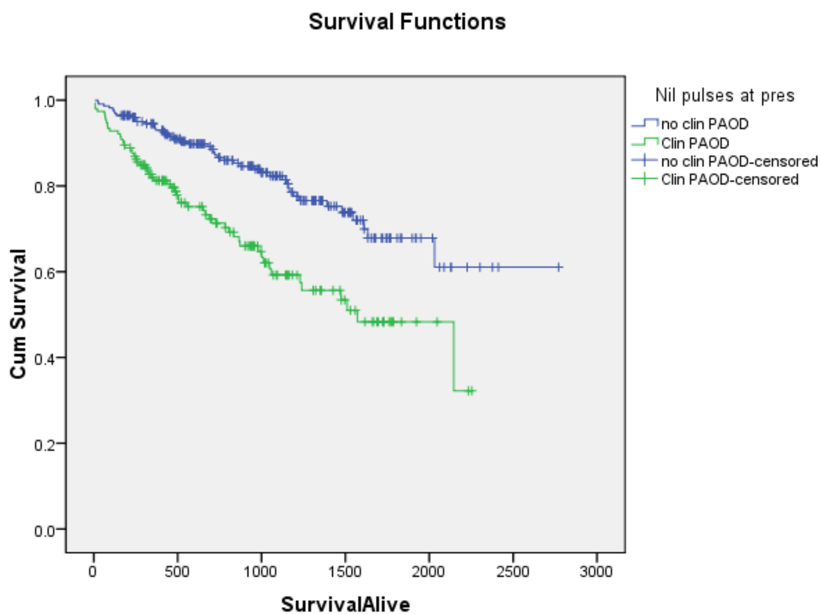
The development of recurrent ulceration was associated with a significantly greater risk of subsequent major adverse event, even if the first presentation had been with superficial ulceration, without infection or ischaemia being present [UoT 0A and 1A] (Figure 2.5).

Figure 2.5: Recurrent ulceration on subsequent major adverse clinical events (p<0.005)



A diagnosis of ischaemia (as defined by the presence or absence of a palpable foot pulse) was associated with a significant reduction in survival (p <0.005) (Figure 2.6). The depth of ulceration and presence/absence of sepsis were also examined using Kaplan-Meier methodology – neither had an effect on survival (these data are not shown).

Figure 2.6: Survival with and without the presence of ischaemia



Time (days)	0	365	730	1095	1460	1825	2190
PAOD (At risk/Events)	153/0	109/28	70/39	39/49	26/51	4/54	2/55
No PAOD (At risk/Events)	225/0	186/14	137/25	94/32	52/39	17/43	1/44

2.4 Chapter summary

These data demonstrate the burden placed on hospital services by the complications of DFU. These data support existing evidence that a structured approach to the assessment of all DFU should include assessment for sepsis and ischaemia and that these features when taken together with the depth of the ulcer at first presentation can identify patients at highest risk of subsequent major adverse events. It also appears from the presented data that even for patients who may appear to present at the benign end of the DFU risk spectrum, a recurrent ulcer indicates a significantly poorer prognosis. This merits further investigation.

A retrospective review of this type will have a number of limitations. A potential confounding issue is the proportion of patients with DFU who may have been lost to follow-up. However given the nature of this patient population and the effectiveness of local electronic health records with the CHI number, it seems unlikely that this would meaningfully influence the presented data. We are unable to make any meaningful comment on the number of patients with septic complications managed in the community.
Furthermore we did not attempt to make a comment on the effectiveness of arterial reconstruction in this patient cohort given the small number of patients who underwent such procedures.

The impact of vascular intervention on an ischaemic limb may be judged by amputation-free survival, graft or vessel patency rates and haemodynamic measures. Defining success following intervention for DFU is more complicated. Given the heterogeneous nature of much of the published work on DFU, the adoption of a standardised clinical assessment is fundamental to allow meaningful systematic review and meta-analysis [Chan 2015]. UoT would seem to be appropriate for this.

Chapter 3 – Patient selection, demographics and the process of skin biopsy

This chapter summarises the process of patient selection (including the ethical approvals that were sought to allow completion) and describes the demographics of the patient groups from which skin biopsies were obtained for the experiments described in chapters 4 and 5.

3.1 Ethical approval and patient selection

Ethical approval to recruit patients to this study was obtained from the West of Scotland Research Ethics Committee (WoSRES) (Research Ethics Committee reference number 13/WS/0008).

A second ethic application was made to the Glasgow Caledonian Skin Tissue Bank to access additional skin samples (this second ethical approval was sought to access skin from the foot).

Patients were considered eligible for inclusion if they presented with critical limb ischaemia as a consequence of peripheral arterial occlusive disease (with or without a concurrent diagnosis of DM) and had been admitted for lower limb arterial reconstruction in the form of infra-inguinal bypass, or major limb amputation and were able to provide informed consent. The clinical decision-making to proceed with intervention was performed according to the symptom pattern reported by the patient, clinical assessment and with the results of cross-sectional angiography (either CTA or MRA). Both elective and non-elective patients were considered to be eligible for inclusion. The only exclusion criterion was cognitive dysfunction that would limit ability to obtain fully informed consent.

Patients undergoing elective orthopaedic foot surgery for mechanical deformity were recruited as a control group (the presence of peripheral arterial occlusive disease was excluded by a combination of clinical history, examination and ABPI assessment – no arterial imaging was performed on this group).

All patients were approached pre-operatively and the study explained. A patient information sheet was provided (the patient information sheet varied depending on the patient group). Fully informed consent was obtained and a consent form signed.

3.2 Methods

Patients were recruited to the study from February 2013 until May 2014.Local and national (PACS) electronic patient record systems were scrutinized through case-linkage using the Community Health Index (CHI) number to complete the basic demographic dataset [Stuart 2013]. A standardised data set was defined which included basic demographic data (age, sex, smoking status). All patients had been screened for DM (a local standard of care in the investigation of patients with symptomatic PAOD). Pharmacological therapy was recorded (statin, antiplatelet and oral hypoglycaemic medication/insulin).

The type of radiological imaging for arterial assessment was documented and the pattern of PAOD recorded. The CHI number was used for this purpose to access nationally archived radiological data.

The presence of sepsis was documented using previously described and validated criteria [Lavery 2007]. Pre-operative therapeutic and prophylactic antibiotic therapy was recorded.

Haemodynamic assessment (ABPI/TBI) was performed in accordance with guidelines published by the Society for Vascular Technology [Cole 2001]. In patients that had previously undergone minor amputation toe pressures were recorded from the most medial remaining digit.

The type of surgical procedure was documented, as was the outcome with specific reference to wound healing.

3.3 Summary of patient demographic data

In total 45 patients were recruited through a combination of the ethical approvals. There were 18 patients with DM and PAOD, 22 patients with PAOD and 5 patients in the control group. The patient dataset is summarised by group in Table 3.1.

The basic demographic data of the different patient groups was broadly as may have been anticipated. There was a male preponderance and smoking was common in the patients with PAOD. None of the orthopaedic control patients had a diagnosis of DM. Of the cohort with DM and PAOD all had Type II DM. Ten of these patients were managed with insulin and the remaining patients were on oral hypoglycaemic medication.

There were 3 patients with soft tissue infection at the time of the primary procedure. In each case Staphylococcus aureus was cultured from wound swabs taken from sites of DFU. All three were prescribed antibiotic therapy (regimes included Tazocin n=1, Vancomycin and Metronidazole n=1 and Benzylpenicillin, Flucloxacillin and Metronidzole n=1).

Patients not receiving pre-operative therapeutic antibiotic therapy were prescribed antibiotic prophylaxis according to local protocol. The vascular surgical patients were prescribed Flucloxacillin and Gentamicin for arterial reconstruction and Flucloxacillin and Metronidazole for major limb amputation. In patients with a Penicillin allergy the Flucloxacillin was substituted for Teicoplanin. The orthopaedic patients received Cefuroxime.

Table 3.1 Summary of patient demographic data

	Control Group (n=5)	PAOD (with DM) (n=18)	PAOD (without DM) (n=22)
Presentation			
Arterial Rest Pain	0	4 (22%)	11 (50%)
Ulceration/Tissue Loss	0	14 (78%)	11 (50%)
Mechanical Deformity	5	0	0
Mean age (range)	50 years (27-71 years)	64 years (51-83 years)	64 years (39-86 years)
Sex (Male)	1 (20%)	13 (72%)	15 (66%)
Pharmacotherapy			
Antiplatelet Agent	1 (20%)	16 (89%)	21 (95%)
Statin	1 (20%)	16 (89%)	17 (77%)
Smoking Status			
Non-smoker	5 (100%)	3 (17%)	2 (9%)
Former-smoker	0	4 (22%)	7 (32%)
Current smoker	0	11 (61%)	13 (59%)
Mean Absolute Ankle Pressure (range)	120mmHg (110-130mmHg)	56mmHg (30-120mmHg)	59mmHg (30-138mmHg)
Mean Absolute Toe Pressure (range)	80mmHg (70-90mmHg)	40mmHg (28-68mmHg)	42mmHg (30-70mmHg)
ABPI			
Normal range (0.9-1.3)	5 (100%)	2 (11%)	0
Occlusive Disease (0.3-0.8)	0	4 (22%)	16 (73%)
CLI (<0.3)	0	5 (28%)	5 (23%)
Incompressible (> 1.3)	0	7 (39%)	1 (4%)
Foot Sepsis	0	3 (17%)	0
Arterial Imaging			
CTA	N/A	6 (33%)	8 (36%)
MRA	N/A	11 (61%)	14 (64%)
DSA	N/A	1 (4%)	0
Pattern of Arterial Disease			
Aorto-iliac	N/A	0	0
Femoral bifurcation	N/A	1 (6%)	0
Femoro-popliteal	N/A	0	11 (50%)
Femoro-popliteal/crural	N/A	11 (61%)	11 (50%)
Crural disease in isolation	N/A	6 (33%)	0
Procedure			
Major Limb Amputation	0	11 (61%)	10 (45%)
Infra-inguinal Bypass	0	7 (39%)	12 (55%)
Forefoot Reconstruction	5	0	0

The mean absolute pressures (ankle and digital) are summarised in Table 3.2. MRA was the preferred method of arterial imaging.

Table 3.2 Description of absolute ankle and digital pressure with reference to the clinical presentation

	PAOD (with DM)	PAOD (without DM)
Rest pain (absolute pressures)		
• **Ankle (range)**	45mmHg (32-54mmHg)	54mmHg (30-82mmHg)
• **Toe (range)**	42mmHg (28-52 mmHg)	44mmHg (30-71mmHg)
Tissue loss		
• **Ankle (range)**	60mmHg (30-120mmHg)	72mmHg (60-130mmHg)
• **Toe (range)**	40 (30-68mmHg)	53mmHg (30-60)

There was one wound failure following major limb amputation in a patient with DM and PAOD necessitating revision to a higher level (transtibial to transfemoral). Of the patients managed with infra-inguinal bypass there was one graft and subsequent wound failure. This patient went on to have a major limb amputation performed. There were no wound failures in the patients with PAOD following surgical intervention on the index admission.

3.4 Process of skin biopsy

Full thickness skin biopsies were taken using a scalpel blade (maximal dimensions 10mm by 5mm) at the beginning of each surgical procedure (all of the skin biopsies were taken by the author). Specimens were harvested from the most proximal and distal incision sites, which was determined by the proposed surgical procedure being performed – for infra-inguinal bypass skin was biopsied from the groin and leg incisions, for major limb amputation skin was biopsied from the foot and then either the leg (transtibial amputation) or thigh (transfemoral amputation) and for the control group skin biopsies were performed from the forefoot (at the level of the 1st metatarsophalangeal joint). Given that ulceration is associated with changes in Cx expression [Wang 2007, Wright 2009, Chin 2011] skin biopsies were taken from anatomical sites distinct from acute/chronic ulceration.

Following this the skin samples were immediately placed in a universal container with 5ml of complete Dulbecco's Modified Eagle Medium (cDMEM) (Lonza, Wokingham, UK) with 0.5 mg/mL Puromycin (Sigma-Aldrich, Gillingham, UK) and 50ml of Foetal Bovine Serum (FBS), which had been refrigerated at 4°C. Following this the sample was placed in ice and transferred immediately to the laboratory.

Subsequent tissue handling was then defined by experiment (the methodologies are described in chapters 4 and 5 respectively).

3.5 Chapter summary

The demographic data described are broadly as would have been expected. The patients with DM/PAOD and PAOD in isolation are comparable in terms of age, sex and smoking status. It is reassuring to see that cardiovascular risk had been considered and addressed appropriately with the administration of an antiplatelet agent and statin for the majority of the patients with PAOD. The control group differs reflecting an entirely different patient demographic from the cohort with arterial disease. No adjustment could be made for this.

The absolute pressures derived at both the ankle and toe, are surprisingly high when compared with validated standards in the world literature [European Working Group on Critical Limb Ischaemia 1992]. It seems likely that there has been consistent methodological error, which has skewed these data.

The patterns of PAOD are as would be expected – patients with DM have a higher burden of more distal arterial disease, with one third of patients having crural vessel occlusive disease in isolation. The pattern of intervention would also seem to reflect this with more of the patients with DM being managed with major limb amputation.

Chapter 4 - Basic Histology and Immunohistochemistry

4.1 Introduction

As has been previously discussed Cx43 is broadly expressed and can be found in all epithelial layers of ectodermal origin [Richards 2005], with more focal distribution in the basal keratinocytes (stratum basale) and with the suprabasal cells (stratum spinosum) demonstrating higher expression [Richard 2000, Brandner 2004]. Cx43(Ser368) has been identified in healthy skin (and noted to be up-regulated in response to injury) [Richards 2004]. In the cardiac myocyte the expression of Cx43 would appear to be down regulated, in response to prolonged hypoxia (more than 5-hours) [Zeevi-Levin 2005], where-as Cx43(Ser368) seems to be rapidly up regulated [Johansen 2011].

In this chapter the expression of Cx43 and Cx43(Ser368) in skin biopsies is explored in vivo, for patients with PAOD with and without DM, as well as from the previously defined control group. Effectively we assess whether there are reproducible changes in either the expression of Cx43 and Cx43(Ser368) such that they could have utility as biological markers of ischaemia in patients presenting with occlusive arterial disease of the lower limb and DFU.

Specific aims were to

i. To explore the presence of Cx43 and compare expression between proximal and distal skin biopsy sites in patients with and without DM undergoing vascular surgical intervention with comparison with a control group without DM or PAOD

ii. To assess whether Cx43(Ser368) could be identified in the same skin biopsies

4.2 Patients and Methods

The process of patient selection and skin biopsy has been summarised in the previous chapter. For these experiments three samples were obtained from each patient group (PAOD with and without DM and control).

4.2.1 Tissue Fixation

On arrival in the laboratory the skin biopsies were removed from the cDMEM and rinsed with Phosphate Buffered Saline (PBS), prior to fixation. The cryobar of the cryostat was activated, cooling to approximately minus 50°C. Mounting discs had OCT placed centrally and were cooled until the OCT was opalescent, following which the tissue sections were mounted. OCT was then placed over the tissue and the disc returned to the cryobar for 5 minutes. When the OCT had set the tissue was sectioned (tissue sectioning was performed at 5 microns). Sectioned tissue was mounted on Colourcoat Adhesion; Silane Coated microscope slides (Cellpath). Each slide had 12 tissue sections mounted from each skin biopsy. On completion slides were placed in a slide box and stored at minus 80°C until ready for processing.

4.2.2 Haematoxylin and Eosin Staining

Slides were allowed to warm to room temperature for ten minutes prior to performing basic histology with Haematoxylin and Eosin staining. This process was performed using a series of Coplin jars. Slides were first placed in Ehrlich's Haematoxylin for fifteen minutes, following which they were transferred to a Coplin jar containing 'Scott's tap water substitute'. After five minutes the slides were rinsed under running water, prior to being placed in another Coplin jar, containing 1% Eosin for a further five minutes. The excess Eosin was blotted from the slide, which was then rinsed under running tap water for a further 5 minutes. Slides were then dipped in acid/alcohol, following which the tissue section was covered with DPX and a glass cover slip. Microscopy was performed and images collected at x10, x20 and x40.

4.2.3 Immunohistochemistry

This technique allows identification of tissue constituents through highly specific antigen/antibody interactions. Two antibodies are required: the primary antibody, which recognised the antigen under investigation and a secondary antibody, conjugated to a chemical (fluorochrome) that fluoresces at a specific wavelength. The procedure was performed in accordance with the standard laboratory protocol (including antibody concentration), the methodology of which is summarised.

Slides were removed from the minus 80°C freezer and placed in ice cold 100% methanol in a Coplin jar to fix the sections on the slides for 10 minutes. Following this the slides were washed with PBS (pH 7.4) to rehydrate the tissue sections. PBS containing 0.1% Titron (Sigma-Aldrich Triton®X-100) was then used to permeabilise the cells (to facilitate entry of the antibodies). Given the viscosity of this solution, it was mixed using a magnetic stirrer. This was left on the slides for thirty minutes. Following this a solution of PBS-T containing 5% milk (Marvel original dried skimmed milk) was placed on the slides for thirty minutes to block any non-specific antibody binding.

Primary antibodies were prepared to a volume of 100µl in diluted in 5% milk-PBS - mouse anti-Cx43 (*Sigma-Aldrich*, St Louis, USA), rabbit anti-Cx43(Ser368) (*Abcam*, Cambridge, UK) and Loricin (*Abcam*, Cambridge, UK) were prepared at 1:500,1:100 and 1:400 respectively. Slides were incubated at 37°C for one hour in a humidity box in darkness. The cells were washed in PBS every 20 minutes for 45 minutes within the humidity box. The secondary antibodies were diluted in 5% milk-PBS - Secondary anti-mouse and -rabbit antibodies conjugated to either AlexaFluor 488 or AlexaFluor 594 (1:500; *Invitrogen*, Paisley, UK) with incubation in the dark for 45 minutes at 37°C in a humidity box. The cells were washed overnight at 4°C in PBS in a humidity box and darkness. The PBS was changed at least twice before putting the plate on a rocker overnight at 4°C. The nuclei were all stained by a subsequent incubation with 4'-6-Diamidino-2-phenylindole (DAPI), a nuclear counterstain (1:1000 in 5% milk-PBS). Finally, the excess PBS was removed from the coverslips by dipping in distilled water and the slides were mounted using Fluorsave (*Calbiochem*, Nottingham, UK)

Immuno-labelled tissue sections were viewed under a x40 oil-immersion lens mounted on a Zeiss 200 Axioscope microscope linked up to a Zeiss LSM 510 META laser scanning

system, using the following settings: Alexa 488 was excited using the Argon laser at 488 nm excitation and emission at 500-550 nm. Alexa 594 was excited with a Helium Neon laser at 543 nm excitation and emission at 600-650 nm.

Images of adequate quality were captured and processed using Adobe photoshop software allowing the creation of an overlay. Following this the images were saved and stored electronically as JPEG files. Analysis is qualitative.

4.3 Results

Skin biopsies were taken from 14 patients for basic histology and immunohistochemistry. A summary of the patients is described in Table 4.1.

Table 4.1 Summary of patient demographic data

	Control Group (n=3)	PAOD (with DM) (n=5)	PAOD (without DM) (n=6)
Presentation			
Arterial Rest Pain	0	0	2
Ulceration/Tissue Loss	0	5	4
Mechanical Deformity	3	0	0
Mean age (range)	49 years (27-71 years)	64 years (57-78 years)	59 years (39-80 years)
Sex (male)	1	4	4
Mean Ankle/Toe Pressure	115mmHg/74mmHg	48mmHg/40mmHg	58mmHg/45mmHg
Foot sepsis	0	1	1
Pattern of Arterial Disease			
Aorto-iliac	N/A	0	0
Femoral bifurcation	N/A	0	0
Femoro-popliteal	N/A	0	4
Femoro-popliteal/Crural	N/A	3	2
Crural	N/A	2	0
Procedure			
Major Limb Amputation	0	3	3
Infra-inguinal Bypass	0	2	3
Complex Forefoot Reconstruction	3	0	0

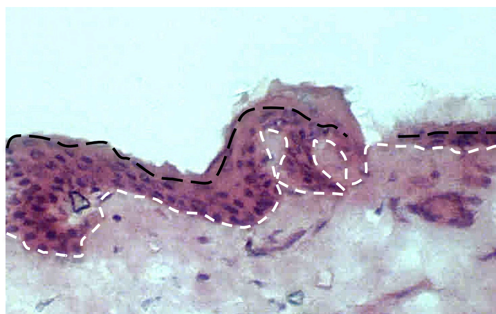
No meaningful immunohistochemistry could be defined from 1 of the control samples. In 5 samples from the PAOD (with and without DM) there was only adequate quality data from either proximal or distal biopsies. There were 6 skin biopsies (PAOD with DM n=3; PAOD n=3) that had good quality proximal and distal biopsy sections available (in 5 of these cases the distal biopsy was taken from the foot).

4.3.1 Basic histology from skin biopsy of the leg and foot

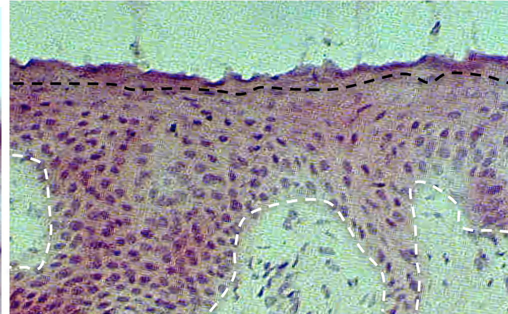
Basic histology demonstrated a standard architectural appearance and confirmed that the sectioning had been perpendicular to the skin surface such that meaningful comment could be made with regard to the immunohistochemistry. An example is provided in Figure 4.1. For cases where sectioning had been inadequate samples were discarded.

Figure 4.1 Haematoxylin and Eosin Staining (x10) of a diabetic patient managed with a major limb amputation, with skin biopsies taken proximally (level of the tibial plateau) and distally (level of the 1st metatarsophalangeal joint)

Proximal skin biopsy



Distal skin biopsy



The proximal skin sample demonstrates thin skin – the stratum lucidum (which is only present in very thick skin and would appear as a homogenous layer between the stratum granulosum and the keratinized layer) is absent. The interrupted white line indicates the stratum basale – the germinal layer of the epidermis. The interrupted black line indicates the stratum granulosum, a layer that is characterized by intracellular granules, which contribute to the process of keratinization. Between these layers is the stratum spinosum that contains cells that are in a process of growth and which are beginning to synthesise keratin. Above these layers is the stratum corneum, which consists of flattened, fused cell remnants that are principally composed of keratin.

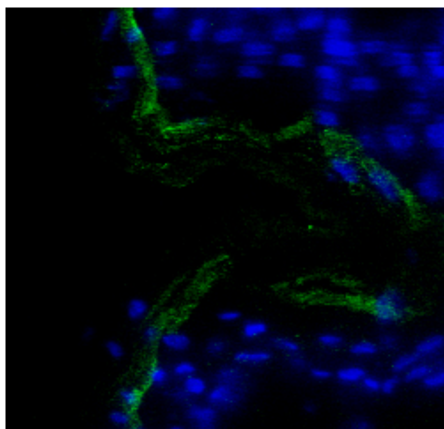
The basic skin architecture appeared to be preserved in all samples. The only difference identified (as demonstrated in Figure 4.1) was with regard to the type of skin (thin skin/thick skin).

4.3.2 Immunohistochemistry from skin biopsy of the leg

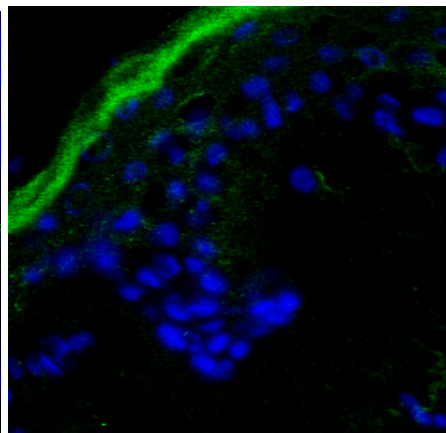
The distribution of Cx43 was comparable when biopsies had been taken from thin skin (Figure 4.2). Cx43 was expressed primarily in the stratum spinosum.

Figure 4.2: Immunohistochemistry for Cx43 (x40) with skin biopsies taken from a diabetic patient undergoing femoro-popliteal bypass. The proximal biopsy was taken from the groin and the distal biopsy was taken from the medial leg. An overlay has been performed. Cx43 is stained green and DAPI nuclear staining (blue) is included.

Proximal skin biopsy



Distal skin biopsy



The spatial distribution of the Cx43 staining demonstrated in this example was consistent with the results from all of the other sections, with Cx43 most abundant in the stratum spinosum. The spatial distribution and expression of Cx43 was similar in the proximal and distal skin biopsies of patients having femoro-popliteal bypass performed. All staining for Cx43(Ser368) from skin biopsies of the thigh or leg was negative – these data have not been shown. Similarly there was no expression of Cx43(Ser368) in the proximal skin biopsies of patients having major limb amputation performed.

4.3.3 Immunohistochemistry from skin biopsy of the foot

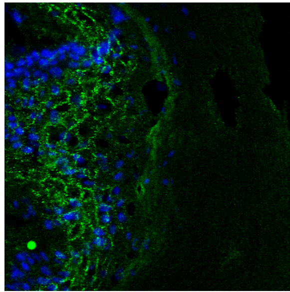
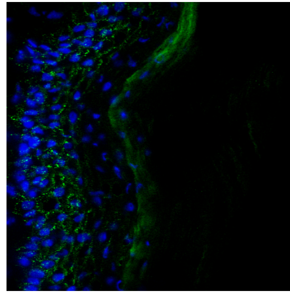
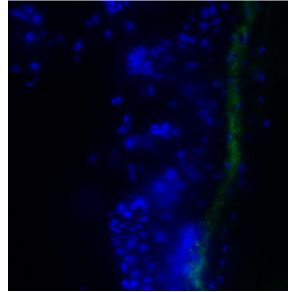
The immunohistochemistry of the skin biopsies taken from the feet of patients generated a different qualitative result pattern, which is summarised in the matrix on the following page (Figure 4.3).

In the patients with PAOD (with or without DM) the ankle and toe pressures were less than 60mmHg and 50mmHg respectively. Cx43 expression and spatial distribution appeared to be consistent with skin biopsies from the thigh/leg and did not appear to be down-regulated in response to ischaemia (irrespective of DM status). Cx43(Ser368) was positively identified with a pattern of expression similar to Cx43 (irrespective of DM status).

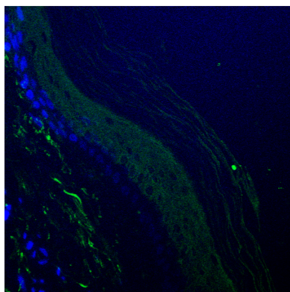
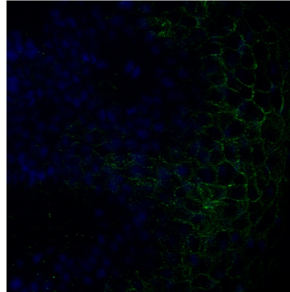
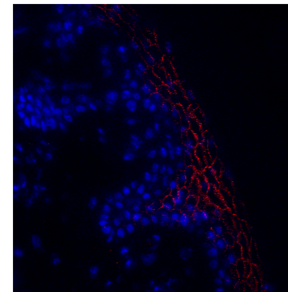
In the control group mean ankle and toe pressures were 115mmHg and 74mmHg respectively. Cx43 expression and spatial distribution was consistent with skin biopsies from the thigh/leg (and the ischaemic feet). Cx43(Ser368) was not identified in any of the control samples.

Figure 4.3: Matrix summarizing the expression of Cx43 and Cx43(Ser368) in skin biopsies from the foot (x40). In these examples Loricrin (a terminally differentiating structural protein found above the granular layer in the epidermis) has also been stained for and a recognised pattern of protein expression identified.

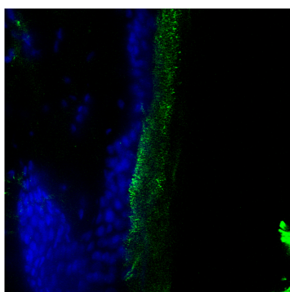
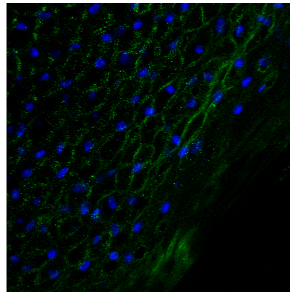
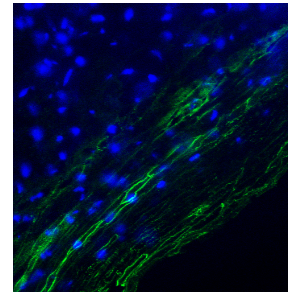
Normal Foot

Loricrin	Connexin 43	Connexin 43(Ser368)
		

Diabetic Ischaemic Foot

Ischaemic Foot

Chapter summary

A standard architectural skin structure has been demonstrated using basic histology. The spatial distribution of Cx43 in human skin is consistent with previously published data, with Cx43 expressed predominantly in the stratum spinosum and with more focal staining in the stratum basale. The presence of significant ischaemia of the foot does not appear to significantly influence Cx43 expression. Cx43(Ser368) was not identified in any of the proximal skin sections nor in the skin sections taken from the feet of the control population. There was marked Cx43(Ser368) expression identified in the sections taken from ischaemic feet. The spatial distribution of the protein in the stratum spinosum is broadly as would be expected from previously published data.

A fundamental conceptual error with the primary study design became apparent following early recruitment of patients having femoro-popliteal bypass performed. As has been described the Cx43 expression was consistent and Cx43(Ser368) was not demonstrated. As has been described discussed, CLI as a consequence of PAOD is primarily a disease of the skin. Therefore it would seem most appropriate to investigate skin of the foot. During the study period there were no patients managed with popliteal to pedal artery bypass recruited to the study. As such all of the biopsies taken from the feet (with the exception of the control group) were from patients undergoing major limb amputation.

Although the quality of the tissue sectioning was good, some samples were discarded because of poor sectioning technique. There were a series of failures of the immunohistochemistry. In some cases tissue samples became dissociated from the slides during the preparation stage. There were some slides where there was inadequate staining of the tissue section, which was presumably a consequence of an unrecognized methodological error. This was addressed by the recruitment of additional patients, such that the dataset could be completed adequately.

Previous researchers have demonstrated Cx43(Ser368) in human skin [Richards 2004]. The absence of Cx43(Ser368) in these sections may have been a consequence of relatively low Cx43(Ser368) expression. Another possibility is technical error with the use of an antibody concentration that was too low. However if this were the case it would suggest that the changes identified in the sections taken from the ischaemic feet are more

significant, with what would therefore appear to be substantial up regulation of Cx43(Ser368).

In two of the study group patients there were wound failures following surgical intervention (failure of amputation n=1, failure of lower limb bypass n=1). Unfortunately the skin biopsies from these patients were used for cell culture, rather than for immunohistochemistry. Given the subsequent clinical course it would have been interesting to explore the proximal skin biopsy for the amputation patient and the distal skin biopsy for the patient who had lower limb bypass performed for Cx43(Ser368).

Cx43 expression was consistent in all of the skin biopsies in terms of qualitative expression and spatial distribution. Consequently it would seem that this protein has no clinical utility as a biomarker for ischaemia in patients with PAOD. The apparent up regulation in ischaemic skin of Cx43(Ser368) is a novel finding. Given that Cx43(Ser368) could not be identified in any of the other skin samples there is an implication that a significant up regulation of protein expression is being seen. As there is consistency between patients with and without DM it seems reasonable to assert from these data that Cx43(Ser368) protein may have utility as a biomarker of ischaemia. Finally these changes would appear to support the assertion that the skin of the foot may be the best place to identify a biomarker for ischaemia.

Chapter 5 – Connexin 43 and Connexin 43(Ser368) expression in-vitro from human keratinocytes and fibroblasts

5.1 Introduction

In this chapter the in-vivo findings described in chapter 4 are further explored with specific reference to the protein expression of Cx43 and Cx43(Ser368) using human keratinocytes and fibroblasts derived from the skin biopsies. As well as examining protein expression following exposure to a hypoxic environment, the potential effects of effect of glycaemia and infection are also explored.

Specific aims were to

i. To investigate the expression of Cx43 and Cx43(Ser368) protein in keratinocytes and fibroblasts in response to a hypoxic challenge

ii. To explore whether Cx43 and Cx43(Ser368) protein expression is influenced by different glycaemic environments

iii. To investigate whether Cx43(Ser368) protein expression is influenced by an infection (in laboratory conditions)

5.2 Patients and methods

The process of patient selection and skin biopsy has been summarised in the previous chapter. For each experiment three samples from each patient group (PAOD with and without DM and control) was considered to be appropriate. The methodologies described below have been validated internally.

5.2.1 Isolation of human fibroblasts and keratinocytes from skin biopsy

Tissue sections were transported to the laboratory where cDMEM was removed from the universal container. The tissue samples were washed in 0.2% Chlorhexidine for 5 minutes. The Chlorhexidine was then discarded and the process repeated. After a further 5 minutes the Chlorhexidine was again discarded and the tissue sample washed with Gentamicin/Amphotericin solution for 5 minutes. This step was then repeated. Using a sterile size-10 blade, excess subcutaneous tissue was excised and disposed of. Following this the tissue was cut into thin strips. Following this the antibiotic solution was discarded and the tissue sections placed in dispase (0.5% w/v in Ca^{2+}/Mg^{2+} free PBS) overnight at $4^{o}C$. The following day the epidermis and dermis were split using forceps and a scalpel blade.

Fibroblast culture

The following day, using sterile forceps the epidermis was peeled from the dermis. The dermis was then cut into small sections using a size-10 blade. These dermal explants were then placed in a T25 Corning flask using sterile forceps. Care was taken to ensure that the explants were widely spaced. At this stage 2ml of cDMEM was added to the flask, which was then placed in an incubator ($37^{o}C$ with 5% CO_2). The dermal explants were allowed to settle for 48 hours following which the media was removed and the explants washed with Ca^{2+}/Mg^{2+} free PBS to remove any debris/non-viable cells. A further 3ml of cDMEM was then added to the flask. The process was repeated on alternate days until a confluent monolayer of fibroblasts had grown.

Keratinocyte culture

The epidermis was then finely chopped until 'slurry' was formed. This was then transferred to the bottom of a universal container with the flat side of the scalpel and 2-3 ml of trypsin added. The solution was left to stand for 2-3 minutes, following which it was agitated with a pipette to create a cell suspension. At this stage 10 ml of Epilife^{TM} (Epilife medium with 1% Epilife-defined growth supplement (formulation proprietary), 10 mg/mL genatamicin, and 0.25 mg/mL amphotericin B [Cascade Biologics, Invitrogen, Paisley, UK]) with 10% chelexed serum was added to the epidermal suspension. A cell strainer was then placed over a fresh universal container and the suspension transferred by pipette. This was then spun down in the centrifuge for 5 minutes at 400 rpm. When this had been done the supernatant was removed by Pipette with care taken not to disturb the cellular pellet and 2ml of Epilife^{TM} with 10% chelexed serum was added to re-suspend the cells. This new suspension was then transferred to T25 flasks that had been prepared with a coating matrix (1ml of the cell suspension was added to each flask) and made up to 5ml with Epilife with 10% chelexed serum. On the second day the media was changed and from that point Epilife^{TM} was used (without chelexed serum). Media was changed on alternate days until the cells became confluent at which time they were split.

All surplus human tissue was discarded in accordance with laboratory protocol.

5.2.2 Splitting cells

Cells were split from either the dermal explants or from flasks when they had grown to either 80% confluence or greater. Media was removed and the cells washed twice with PBS to remove any non-viable cells. Trypsin was then added to the flask (1ml to the dermal explants in T25 flasks and 2ml to the cells in T75 flasks). The flasks containing the Trypsin were then returned to the incubator for three minutes, following which either 5ml of Epilife^{TM} or cDMEM were added (for keratinocytes and fibroblasts respectively). The cells were placed in a Universal container and then placed in the centrifuge for 5 minutes at 10,000g. The supernatant was discarded, the pellet re-suspended and the cells re-plated (to expand the cell colonies cells were re-plated in T75 flasks).

5.2.3 Freezing Cells

Keratinocytes and fibroblasts were harvested on the second passage after they had grown to confluence in T75 flasks. Trypsin was added to the flask (2ml), which was then replaced in the incubator for three minutes, following which 5ml Epilife^{TM} (keratinocytes) or cDMEM (fibroblasts) was added. The solution was removed from the flask and added to a Universal container for centrifugation (5 minutes at 10,000g). The supernatant was discarded and the pellet re-suspended in freezing serum prior to transfer to a cryovial. The cells were then slowly frozen to minus 70°C and thereafter stored in liquid nitrogen.

5.2.4 Cell Challenges

A variety of different cell challenges were experimented with. In each case cells were grown to approximately 80% confluence in 6-well plates prior to challenge. Cells were used on the second or third passage.

5.2.4.1 Hypoxia

Cells were challenged in a Hypoxia Chamber (Stemcell^{TM} Technologies). The chamber was prepared in accordance with the manufacturer's instructions for use. Plates of cells were placed in the chamber along with a petridish containing 20ml of distilled water, following which the chamber was closed. Gas was then allowed to flow into the chamber at 25l/minute for 4 minutes in accordance with the manufacturers instructions for use, following which the chamber was sealed. Cells were challenged in a 1% Oxygen/5% Carbon Dioxide/Nitrogen environment. Fibroblasts were challenged in this environment 12, 24 and 48 hours. Keratinocytes were challenged for 6, 12 and 24 hours (cell death was seen with the keratinocytes if they were left in these conditions for 48 hours).

5.2.4.2 Peptidoglycan

Peptidoglycan (PGN) is a polymer that is found outside the plasma membrane of most bacteria, forming a substantially thicker layer in gram-positive bacteria. In these experiments challenge with PGN was designed to mimic a Staphylococcal infective insult – gram-positive aerobic bacteria are the most common organisms isolated from DFU [Lipsky 1990, Tentolouris 1999].

Prior to challenge with PGN cells were treated for 60 minutes with culture media without antibiotics. PGN was then added at 10µl/ml. Cells were harvested at 0, 6 and 24 hours following the challenge.

5.2.4.3 High and Low Glucose Conditions

To prepare media for high and low glucose conditions, a 1% stock of Bovine Serum Albumin (BSA) was first made in both low glucose and high glucose, warmed serum free DMEM (sfDMEM). This was then filter sterilised. Using this BSA stock 100ml of low and high glucose DMEM with 0.1% BSA was prepared.

A 10µM insulin stock was prepared by adding 58.8µl to 9.9412ml of low glucose sfDMEM with 0.1% BSA. Low and high insulin glucose media (10mls) was then prepared as described in the following tables.

Table 5.1 Preparation of high and low glucose media

Metabolic Conditions	Glucose Concentration (mM)	Insulin Concentration (nM)	sfDMEM + 0.1% BSA (ml)	Insulin Stock (µl)
Low Glucose	5.5	1	9.999	1
High Glucose	25	10	9.633	10

Cells were maintained in standard conditions in 6-well plates until ready for experimentation. Cells were treated for five days in either high or low glucose environments. The cells were washed daily with PBS and the media was changed.

5.2.5 Protein Harvest

Cells were cultured in 6-well plates for protein extraction and then challenged as required. After treatment the media was removed (the supernatant was collected in eppendorf tubes and frozen at minus 20°C), following which the samples were washed twice with PBS. The cells were then lysed using 100µl of ice-cold lysis buffer and harvested by scraping using the rubber plunger of an insulin syringe. The cell lysates were then added to ice-cold eppendorf tubes and sonicated three times at 15MHz for ten seconds. Samples were frozen at minus 70°C until required.

Previous local data and previously published data suggested that 20mcg of protein should be adequate for Cx43 staining. Work from previous authors suggested that a similar protein concentration should allow adequate staining of Cx43(Ser368).

5.2.6 Bradford Protein Assay

The Bradford protein assay, a spectroscopic analytical procedure based on the Lowry assay, which measures the reaction of proteins with alkaline copper tartarate and Folin reagent was used to determine the protein concentration from each sample. The BioRad system (*BioRad*, UK) was utilised.

In summary, 5µl of each protein sample was added in duplicate to a 96-well plate. This was then mixed with 25µl of Reagent A + S (1ml of Reagent A and 20µl Reagent S) following which 200µl of Reagent B was added. Samples were then incubated at room temperature on a plate rocker for 15 minutes to allow colour to develop. Following this a plate reader was used to calculate absorbance. Protein concentration was then calculated using a linear regression model generated using a known concentration solution of BSA (0 to 1mg/ml) prepared in the same buffer used for protein extraction and Microsoft Excel.

5.2.7 Western Blot Analysis

Western Blot analysis was completed using pre-cast NuPAGE^{R} Bis-Tris mini gels (Novex by life technologies). These were removed from the packaging and rinsed with deionised water. The loading combs were then removed and gels transferred into the running apparatus, following which the running buffer (50ml 20X NuPage^{R} was added to 950ml deionised water to prepare 1X SDS running buffer). The protein ladder (10µl) and protein samples (well volume 35µl) were then loaded using a pipette. For Cx43 20mcg of protein was loaded and for Cx43(Ser368) 60-80mcg of protein was loaded. Each protein sample was loaded in two wells. The gels were run at 200V for 1 hour or until all the protein marker band has run off the bottom of the gel. During this time TBS and TBST were prepared.

On completion the gel cassettes were split open and the gel removed. This was then placed in the iBlot apparatus the iBlot stack was the added (the membrane had been soaked in

deionised water). On completion the blotting membrane and plastic tray were removed. The blot was washed with Ponceau S solution (to check for successful transfer of protein to the membrane) and then rinsed with deionised waster.

The membrane was then placed in a 50ml universal container and blocked with 5% milk TBS for 60 minutes. A solution of the primary antibody [Cx43 (1:2000), Ser368 (1:500) and GAPDH (1:4000)] was made up to 5ml using 5% Milk TBS. The membrane was allowed to incubate overnight at 4°C.

The following day the primary antibody (was removed and stored - 0.02% of Azide [3.75 μL of 20% Azide in 5 mL] was added for the purpose of to preservation. And the antibody stored in the fridge.

The membrane was then washed at 10-minute intervals with TBS-T for 45 minutes, before being blocked again with 5% Milk TBS for 30 minutes. At this point 5 ml Secondary antibody (Goat anti-mouse- BioRad, Goat anti-Rabbit- BioRad attached to Horse Radish Peroxidase 1:2000 dilution. In 5% milk TBS (10 μL in 20 mL) to the membrane, which then allowed to incubate for 60 minutes at room temperature. The membrane was then washed at 20-minute intervals with TBS-T for 1 hour. The membrane was then washed with PBS (Sigma) for 10-minutes, prior to the addition of the ECL reagents (1.5 ml reagent A plus 1.5 ml reagent A) and the membrane allowed to incubate for 1 minute.

At this point blots were wrapped in cling film and analysed using Odyssey Imager acquisition system (LI-COR) using chemiluminescence and fluorescence. A digital representation of protein expression was generated, which allowed for data analysis.

The blot was then re-probed for the protein standard to assess protein loading (in this case GAPDH).

Following analysis the blots were re-wrapped in cling film and stored in PBS at 4°C, such that they could be re-probed if necessary.

5.2.8 Statistical Analysis

Semi-quantitative analysis was performed. The mean signal identified from paired protein samples for Cx43 and Cx43(Ser368), was normalized and expressed graphically using Excel with standard error bars.

5.3 Results

There were 23 patients recruited for skin biopsy with a view to culture of both keratinocytes and fibroblasts. Cell lines could only be cultured from 8 patients (in 5 cases fibroblasts were cultured and in 3 cases keratinocytes were cultured). Fibroblasts proved slightly easier to culture and maintain.

The demographic data of these patients is summarised in Table 5.2.

Table 5.2 Summary of patient demographic data

	Control Group (n=3)	PAOD (with DM) (n=2)	PAOD (without DM) (n=3)
Presentation			
Arterial Rest Pain	0	1	2
Ulceration/Tissue Loss	0	1	1
Mechanical Deformity	3	0	0
Mean age (range)	43 years (27-54 years)	74 years (64-83 years)	70 years (66-72 years)
Sex (male)	1	2	1
Mean Ankle/Toe Pressure	120mmHg/82mmHg	65mmHg/52mmHg	48mmHg/43mmHg
Foot sepsis	0	0	0
Pattern of Arterial Disease			
Aorto-iliac	N/A	0	0
Femoral bifurcation	N/A	0	0
Femoro-popliteal	N/A	0	0
Femoro-popliteal/Crural	N/A	1	2
Crural	N/A	1	1
Procedure			
Major Limb Amputation	0	2	1
Infra-inguinal Bypass	0	0	2
Complex Forefoot Reconstruction	3	0	0

5.3.1 Protein extraction

The quantity of protein extracted per well was poor. Given the relatively low protein yield some consideration was given to making the gels to run the Western-blot analysis as these could be created with larger wells, which would accommodate a greater volume. However this process was to prove time consuming and the quality of the blots (methodology of gel construct not described and data not shown) was not as good as the commercially available gels. This issue was overcome by pooling the protein harvested from 3-wells, which provided a low, but usable quantity of protein to allow the Western blot analysis to be performed.

Figure 5.1 and Table 5.3 demonstrate the process (and result of) a Bradford protein assay with protein calculation.

Figure 5.1, Table 5.3 and Table 5.4 Bradford protein assay from human keratinocytes subjected to hypoxic challenge

Table 5.3 Standard protein absorbance data

Volume BSA (mg/ml)	Absorbance at 595nm
0	0.0169
0.25	0.044
0.5	0.092
0.75	0.161
1	0.191

Figure 5.1 Linear regression model (Bradford protein assay)

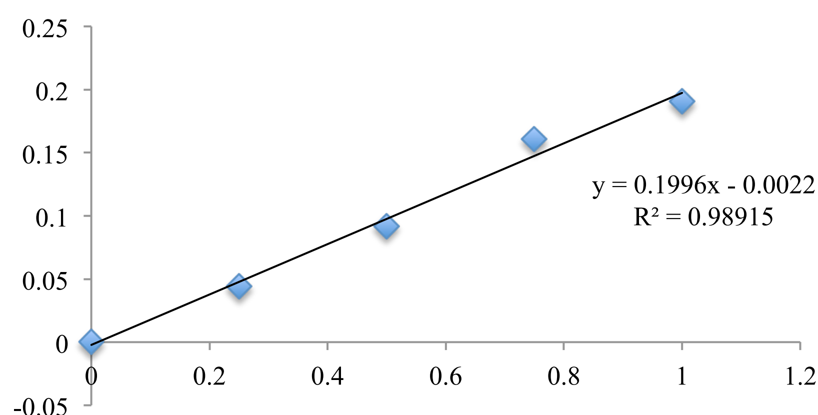


Table 5.4 Derived protein concentrations

	Control (0 hour)	6 hour	12 hour	24 hour
Absorbance at 595nm	0.586	0.748	0.906	0.719
Protein in sample (mg/ml)	2.9	3.7	4.5	3.6
Volume required (μ/l) for				
• 20mcg Protein	6.8	5.3	4.4	5.5
• 80mcg Protein	27.2	21.3	17.6	22.2

5.3.2 Expression of Cx43 and Cx43(Ser368) from human fibroblasts and keratinocytes following hypoxic challenge

As described fibroblasts were cultured from 5 patients (PAOD/DM n=1, PAOD n=2 and control n=2) and protein extracted for analysis (Cx43 n=3 and Cx43(Ser368) n=3). Fibroblast data following hypoxic challenge is summarised in Figures 5.2, 5.3 and 5.4.

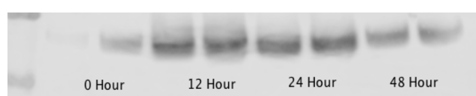
Figure 5.2 Western blot analyses for Cx43 and Cx43(Ser368) - Control

A and B: Cx43 expression in fibroblasts with GAPDH control following hypoxic challenge

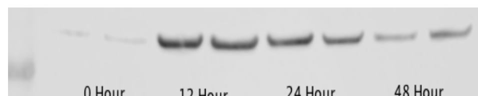
C and D: Cx43(Ser368) expression in fibroblasts with GAPDH control following hypoxic challenge

E and F: Data expressed as a mean for each time point for Cx43 and Cx43(Ser368)

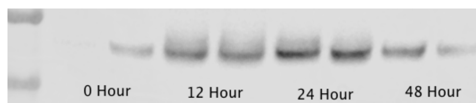
(A) Cx43 expression



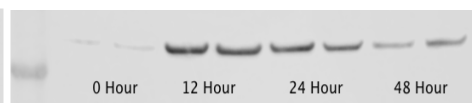
(B) GAPDH expression of blot A



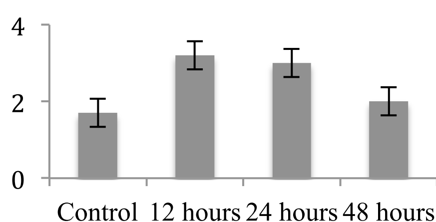
(C) Cx43(Ser368) expression



(D) GAPDH expression of blot C



(E) Cx43 expression



(F) Cx43(Ser368) expression

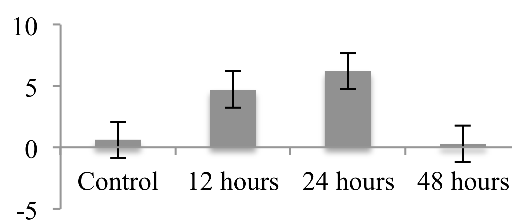
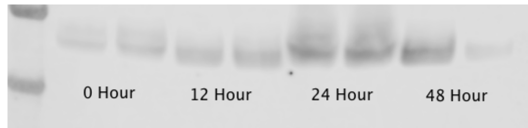
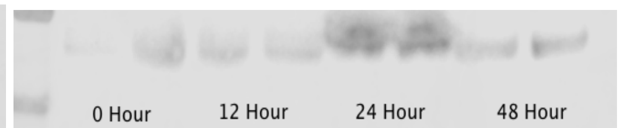


Figure 5.3 Western blot analyses for Cx43 and Cx43(Ser368) – PAOD/DM
A and B: Cx43 and Cx43 expression in fibroblasts following hypoxic challenge
C and D: Data expressed as a mean for each time point for Cx43 and Cx43(Ser368)

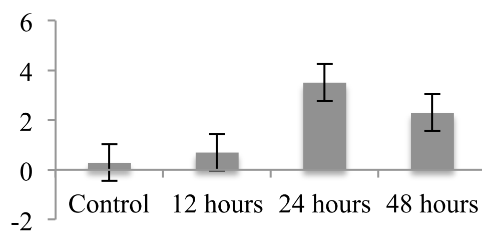
(A) Cx43 expression



(B) Cx43(Ser368) expression



(C) Cx43 expression



(D) Cx43(Ser368) expression

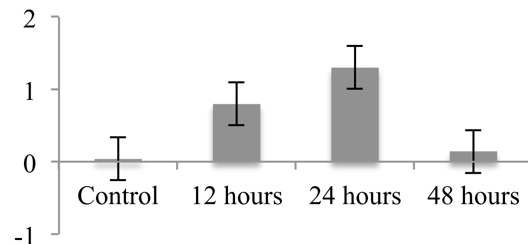
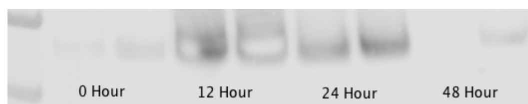
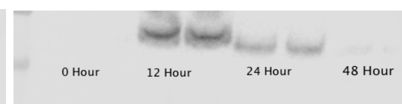


Figure 5.4 Western blot analyses for Cx43 and Cx43(Ser368) – PAOD
A and B: Cx43 and Cx43 expression in fibroblasts following hypoxic challenge
C and D: Data expressed as a mean for each time point for Cx43 and Cx43(Ser368)

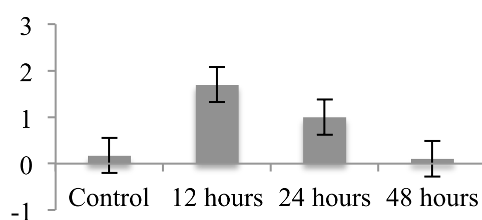
(A) Cx43 expression



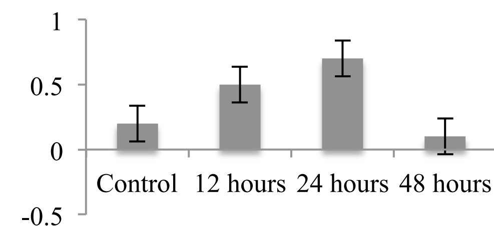
(B) Cx43(Ser368) expression



(C) Cx43 expression



(D) Cx43(Ser368) expression



In these experiments there appeared to be consistent up-regulation of both Cx43 and Cx43(Ser368) at 12 and 24 hours irrespective of the cellular phenotype.

Keratinocytes were cultured from 3-patients (PAOD/DM n=1, PAOD n=1 and control n=1) and protein extracted for Cx43 and Cx43(Ser368) analysis. In keratinocytes the trend was for induction of Cx43 between 6 and 12-hours, with a similar pattern of induction demonstrated for Cx43(Ser368). The DM and non-DM keratinocytes did not seem to behave differently in terms of Cx43 and Cx43(Ser368) expression. These data are summarised in Figure 5.5, 5.6 and 5.7.

Figure 5.5 Western blot analyses for Cx43 and Cx43(Ser368) - Control
A and B: Cx43 expression in keratinocytes following hypoxic challenge
C and D: Data expressed as a mean for each time point for Cx43 and Cx43(Ser368)

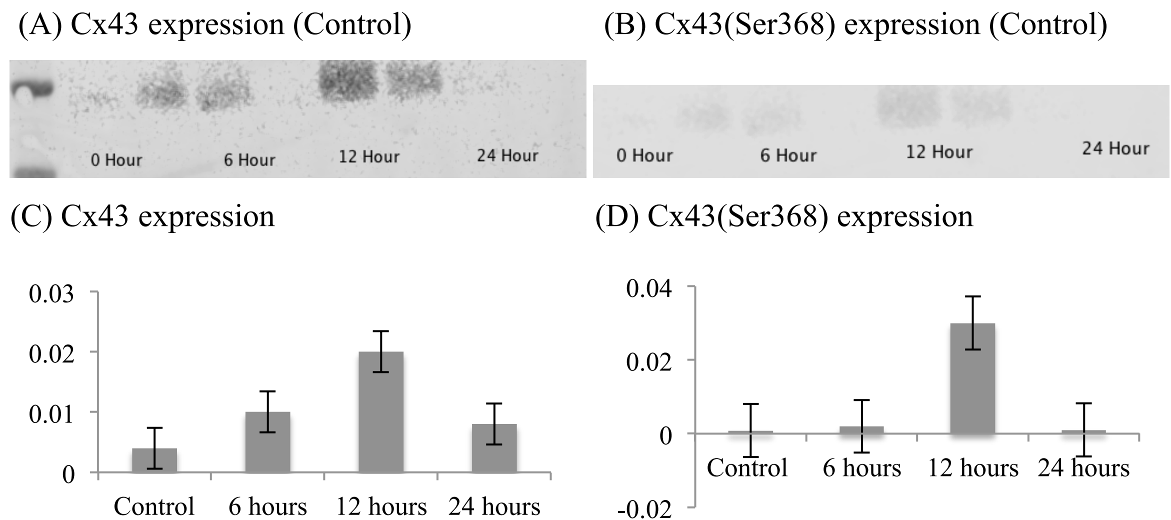


Figure 5.6 Western blot analysis for Cx43 and Cx43(Ser368) – PAOD/DM

A and B: Cx43 and Cx43 expression in keratinocytes following hypoxic challenge

C and D: Data expressed as a mean for each time point for Cx43 and Cx43(Ser368)

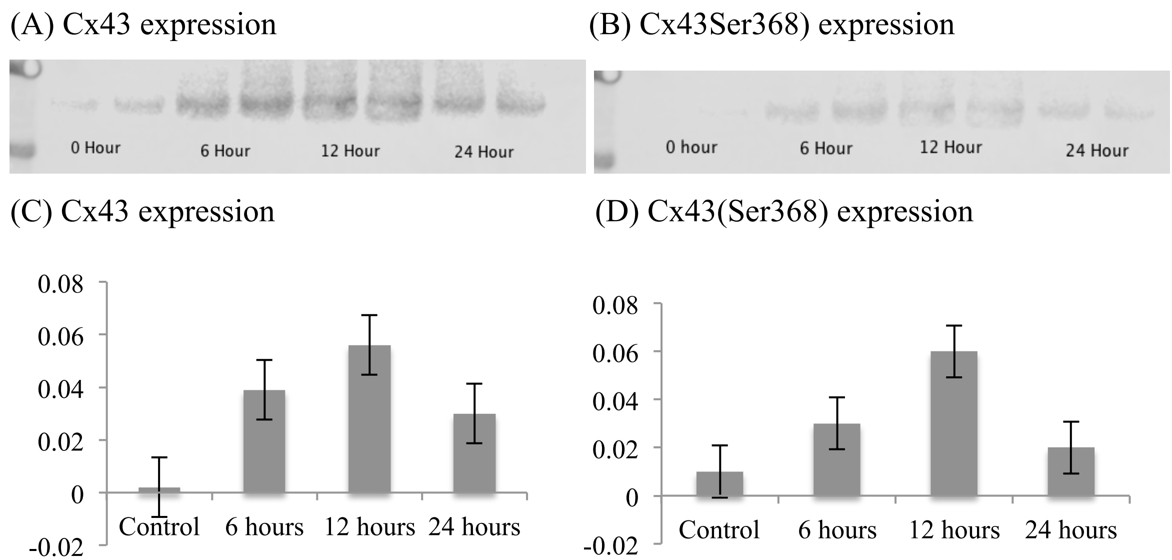
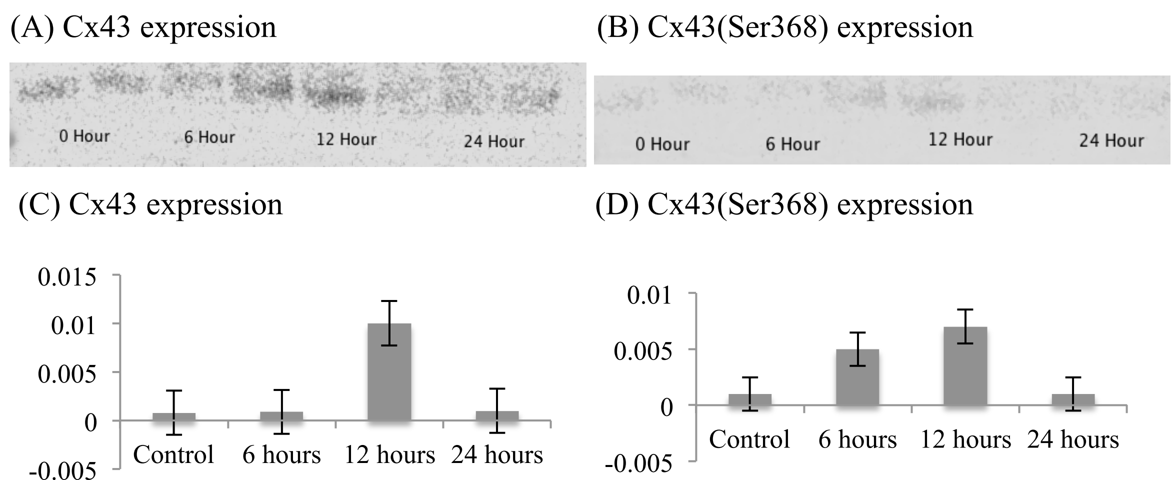


Figure 5.7 Western blot analyses for Cx43 and Cx43(Ser368) – PAOD (GAPDH control not shown)

A and B: Cx43 and Cx43 expression in keratinocytes following hypoxic challenge

C and D: Data expressed as a mean for each time point for Cx43 and Cx43(Ser368)



5.3.3 The influence of high and low glucose environments on the expression of Cx43 and Cx43(Ser368) – Failed experiment

This experiment was attempted with fibroblasts and keratinocytes using the protocol described above, which had been validated by other scientists within the laboratory.

When the cells were 80% confluent in 6-well plates treatment with the high and low glucose solutions was started. There was no evidence of further cell proliferation during the 5-days of treatment and in some cases cell death was witnessed.

The amount of protein that was extracted from the cells was so low (results of Bradford protein assays not shown) that Western Blot analysis could not be performed.

5.3.4 Expression of Cx43 and Cx43(Ser368) from human fibroblasts following peptidoglycan challenge

The expression of Cx43 and Cx43(Ser368) in human fibroblasts following exposure to PGN was explored using cells from three patients (fibroblasts from PAOD/DU n=1; fibroblasts from control n=2).

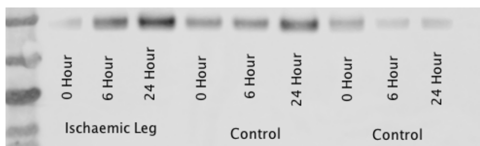
Figure 5.8 Cx43 and Cx43(Ser368) expression in human fibroblasts following peptidoglycan challenge

A and B: Cx43 expression in human fibroblasts with GAPDH control following PGN challenge

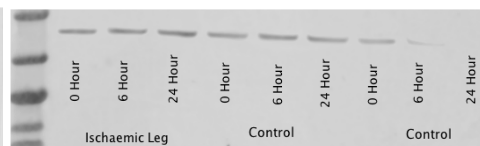
C and D: Cx43(Ser368) expression in human fibroblasts with GAPDH control following PGN challenge

E and F: Data expressed as a mean for each time point for Cx43 and Cx43(Ser368)

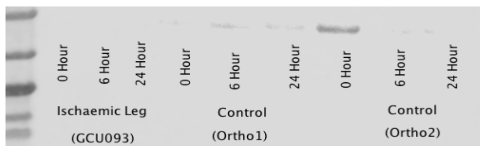
(A) Cx43 expression



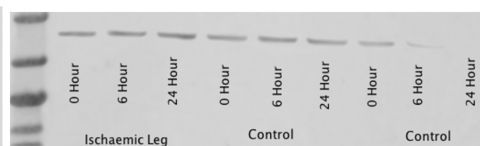
(B) GAPDH expression of blot A



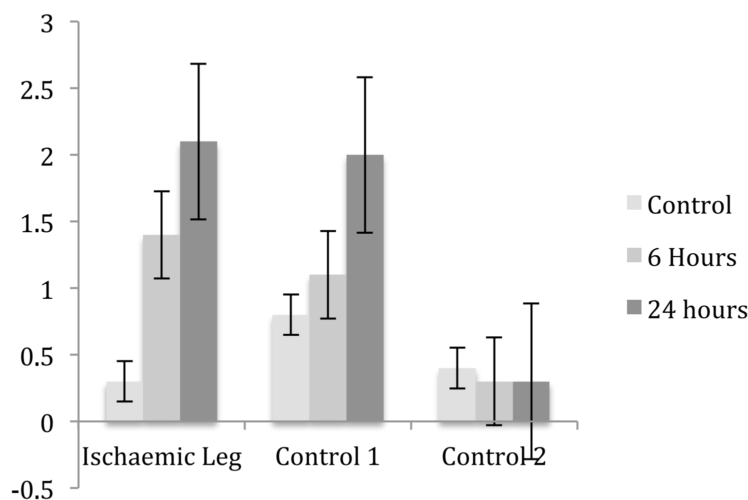
(C) Cx43(Ser368) expression



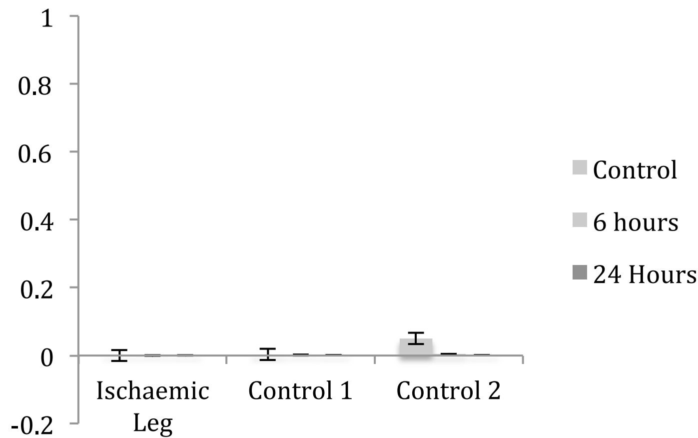
(D) GAPDH expression of blot C



(E) Cx43 expression



(F) Cx43(Serine 368) expression



These data suggest that Cx43 is induced at 6 and 24 hours following PGN challenge. There was no evidence of Cx43(Ser368) phosphorylation being induced.

(There is no comparable data available for Cx43 and Cx43(Ser368) expression in human keratinocytes following PGN challenge, because of the difficulties encountered culturing and maintaining these cell lines from the tissue samples collected).

5.4 Chapter summary

These data seem to imply that *in-vitro* the protein expression of Cx43 and Cx43(Ser368) derived from both human keratinocytes and fibroblasts are upregulated by a hypoxic challenge. PGN challenge seems to induce Cx43, but had no influence on the expression of Cx43(Ser368). It is not possible to comment on the effect of metabolic dysfunction.

A number of practical difficulties were encountered. Cell culture proved to be challenging and in many cases neither fibroblasts nor keratinocytes could be cultured despite strict adherence to the pre-defined local protocol. Protocols developed by other scientific teams were scrutinized, but despite minors modification of the cell harvest protocol no clear solution to this problem was identified during the research.

Difficulty with cell culture should have been anticipated as in approximately 50% major limb amputation was being performed in the context of critical limb ischaemia. Presumably the severe distal ischaemia had an impact on the subsequent behaviour of the keratinocytes and fibroblasts. It is unclear as to why it proved so difficult to culture cells from the proximal skin biopsy sites. Infection did not seem to be an issue. It seems likely that issues with the skin was compounded by a significant methodological error at some point in the process contributed to failed cell culture attempts.

In cases where fibroblasts and keratinocytes were cultured, the time from skin biopsy to experiment (performed when the cell line was 80% confluent) was significant – the mean time recorded for this process was 28-days. The long time from cell harvest to experiment persisted throughout the duration of the study.

Another potential issue has been with the choice of GAPDH as a 'housekeeping protein'. The protein worked well and has implied consistent protein loading (in the PGN experiments), however there is mixed evidence about its usefulness, with some authors suggesting that GAPDH may have limitations as a reference protein [Ferguson 2005]. Specifically there is some evidence that hypoxia may influence GAPDH expression and although this evidence is primarily based upon RNA analyses [Zhong 1999, Bär 2009] the pattern of GAPDH expression highlighted in Figure 5.2 (blots B and D) suggest that this is also the case for protein expression. Attempts were made to re-probe the blots with α-Tubulin/β-Actin without success.

It was unclear as to why the keratinocytes and fibroblasts behaved as they did in the high and low glucose media. Given the difficulty with primary cell culture and the limited availability of cells, these experiments could not be repeated.

There are two potential conclusions that we can make from these data. Firstly it would appear that Cx43(Ser368) protein expression might have utility as a biomarker for ischaemia in patients with PAOD and DFU, with apparent up-regulation of the protein at 6 and 12 hours. Secondly Cx43 protein expression appears to be up-regulated by PGN challenge and given that infection is a significant potential issue in patients with DFU this would appear not to have clinical utility. However these conclusions must be interpreted with caution given the potential confounding issue of using GAPDH as the protein standard. These *in-vitro* results require further validation.

Chapter 6 – Discussion and Conclusions

6.1 Introduction

DFU is a challenging condition that will be encountered more frequently as a consequence of the significant increase in prevalence of DM with an aging population. In order to improve patient outcomes, identification of the at risk patient population may allow the multidisciplinary team to target resource and intervention to appropriately selected groups. For this to be effective, clear and unambiguous communication between members of the multidisciplinary diabetic foot care team is essential.

In patients with neuroischaemic DFU the clinician is frequently challenged as to the appropriateness or otherwise of arterial reconstruction (endovascular or surgical). This is inextricably linked to the challenges of patient assessment and uncertainty about the impact of intervention for this pattern of ulceration and PAOD. Ongoing clinical trials such as BASIL-2 (NIHR Health Technology Assessment grant; project number 12/35/45) a multi-centre randomized trial comparing the outcome of endovascular and surgical intervention for patients with infra-geniculate PAOD (with or without concurrent femoro-popliteal segment PAOD) may help inform opinion with regard to procedure choice for an individual patient, but will not help with the patient selection.

Consequently two questions remain – Firstly can we identify the patient population with DFU who will benefit from intervention and secondly will we modify the natural history of the disease process with intervention. Unless the first question is meaningfully answered, truly understanding the appropriateness and effectiveness of our interventions will remain uncertain.

Identification of a biomarker of ischaemia in this patient group could offer potential benefit in terms of guiding clinical decision making.

6.2 The effectiveness of University of Texas classification to describe diabetic foot ulceration

These present data indicate that the clinical features defined by the UoT classification on first presentation, allow identification of patients most at risk of the major adverse events associated with DFU. Increasing ulcer depth and the presence of ischaemia and/or infection are independently associated with a significant increase in the requirement for major limb amputation and arterial reconstruction. These findings are consistent with other data demonstrating strong links between clinical characteristics and the likely clinical course of an individual ulcer [Jeffcoate 1993, Lavery 1996, Mills 2014]. Deeper ulcers and more advanced stage are also associated with an increased requirement for secondary care admission with septic complications. Finally, we have demonstrated that the development of recurrent ulceration is associated with an increased risk of major adverse clinical events even in the group presenting with apparently benign primary ulceration.

To be effective a scoring system must be easy to apply, reproducible, identify high-risk cases and facilitate communication between different members of the multidisciplinary healthcare team [Teasdale 1974]. Clinical examination, defined as the presence or absence of a pedal pulse would seem to reliably identify patients with significant large vessel occlusive atherosclerotic arterial disease. Even with the potential shortcomings of such a basic categorical test, the high-risk foot seems to be identified [Byoko 1997]. A standardised clinical classification of sepsis has been defined [Lavery 2007], but there are limited data available to validate how this is applied for the various DFU scoring systems, creating the potential for inter-observer variability. However, despite these apparent limitations only eleven limbs in this series were "upgraded" during the follow-up period. This implies that despite the relatively crude nature of the clinical assessment UoT may appropriately classify DFU.

The strengths of UoT classification are simplicity and an entirely clinically based assessment. The fundamental components of UoT have been embedded into the 'Wound, Ischaemia and Foot Infection' (WIfI) classification system described by the Society for Vascular Surgery (Mills 2014). This scoring system can be utilised to assess patients with and without DM. In this scoring system the diagnosis of ischaemia is based upon haemodynamic/perfusion measurements, rather than clinical examination. Retrospective validation has confirmed that this scoring system has prognostic utility (Cull 2014).

Although the addition of a haemodynamic measure of perfusion may be beneficial (the data may be valuable, particularly for research and measuring outcomes) it increases the complexity of the assessment process. Rather than engaging the primary healthcare team this could delay referral to and discussion with tertiary care. Furthermore there is no current evidence that assessment using WIfI confers any additional benefit over UoT in terms of identification of the 'at risk' limb.

Any clinician assessing a patient presenting with DFU needs to consider whether sepsis is present and, if so whether urgent intervention to control sepsis is required. Similarly the extent to which large vessel occlusive arterial disease may be contributing to the presentation must be considered. Classifying the ulcer by the UoT system leads the clinician through this assessment process and generates prognostic information for the ulcer, limb and patient.

There remains debate about the impact of some interventions on the natural history of DFU and this merits further investigation [Jude 2001*]. Two fundamental issues complicate defining the effectiveness of intervention – the heterogeneous nature of the patient population with variation in clinical assessment and variation in outcome reporting following intervention. These issues limit meaningful meta-analysis. Minor amputation rates are often reported, but the relationship of this to the outcome of the limb is weak in both this and previously series [Prompers 2008]. Minor amputation may often be required to achieve adequate surgical control of sepsis and as such probably represents a difficult and misleading measure of the quality of care in patients with DFU [Jude 2001*]. A rate of major limb amputation of approximately 5% per year following presentation with DFU has been described [Jude 2001, Engelhardt 2008, Chan 2015]. This clinical end-point is an attractive metric given its binary nature (as is amputation-free survival), but there are adverse events other than limb loss or death, which have significance to patients and healthcare systems. The disease process continues in other measurable ways and potential metrics to assess the effectiveness of an in intervention could include the number and duration of subsequent septic episodes (requiring admission for conservative and/or operative management), time to healing (of ulcers/surgical wounds) and ulcer recurrence. More difficult to assess are measures of mobility and independence following intervention. All of these features directly influence quality of life for patients with DFU [Elgzyri 2013].

Relevant to surgeons in particular, but all members of the multidisciplinary healthcare team is a reminder that one year after 'successful' intervention with limb salvage, 25% of patients will have unhealed surgical wounds or index ulcers [Chung 2006, Söderström 2008]. Similarly, when assessing the impact of any of the components of the multidisciplinary care package it is important to note that some ulcers will heal when treated conservatively (Elgzyri 2013). To further confuse matters, many ulcers fail to heal despite a functioning graft and there are few data that describe the impact of limb salvage surgery or surgical management of sepsis on recurrent infection rates, mobility, time spent in hospital or death due to sepsis. Given the nature of the disease process, even successful interventions are unlikely to confer permanent protection from all future major adverse clinical events, and perhaps a modification of the natural history of the disease is the best that can be hoped for and outcomes assessed accordingly [Chan 2015].

6.3 Connexin 43 and Connexin 43 (Serine 368) expression and in-vivo and in-vitro

The expression of Cx43 in the tissue sections was similar in both the control and study groups (including the proximal skin biopsies from patients having both lower limb bypass and major limb amputation) and is consistent with previously published data. Cx43 expression from the feet of these was also comparable to the patterns of expression identified in the skin biopsies of the leg (although morphologically the skin of the foot differs from that of the leg – thick skin and thin skin respectively). This is consistent with previous findings [Richard 2000, Brandner 2004, Richards 2005].

The apparent up regulation of Cx43(Ser368) in the skin biopsies taken from the ischaemic feet was a novel finding. Previous work [Richards 2004] has described the presence of Cx43(Ser368) in the stratum spinosum of normal skin, although was not demonstrated in this study. Up regulation of Cx43(Ser368) has been described as a consequence of injury, but these changes were not seen for approximately 6 hours and did not peak until 24 hours [Richards 2004]. Although by definition skin biopsy results in controlled epithelial trauma, it would seem unlikely that this in isolation could have generated such significant changes in Cx43(Ser368) protein expression. The skin biopsies from this study were taken directly to the laboratory on ice for fixation. These changes were not identified in any of the skin samples from the legs of patients (with and without DM), nor was it demonstrated in any of the control samples. Consequently it would seem likely that the changes in protein expression here are a true reflection of Cx43(Ser368) expression in the foot at the time of skin biopsy.

The in vivo expression of Cx43(Ser368) was independent of a diagnosis of DM, implying that the changes reflect the ischaemic environment rather than the metabolic changes associated with glycaemia. In vitro is difficult to ascertain whether the protein expression from the human fibroblasts and keratinocytes was truly representative of the DM or non-DM state. The cells were cultured under standardised laboratory conditions and the mean time from cell harvest to experiment was significant. Whether these cells retained a 'DM phenotype' through this process is uncertain. At an organ level the concept of 'glycaemic memory', a term that describes the delayed effects of previous hyperglycaemia on diabetic complications in later life, irrespective of subsequent improvements in glycaemic control has been discussed [UKPDS 1998, DDCT 2005]. Whether this concept is reflected at an individual cellular level in the human fibroblasts and keratinocytes cultured for these

experiments is less certain. There is some evidence from wound healing models demonstrating differences in migration of human fibroblasts and keratinocytes (derived from foreskin and skin biopsies) from DM and non-DM donors, even if the cells were maintained in euglycaemic conditions if they were investigated at an early passage [Pollock 2011]. If the cells in this experiment have similar characteristics to those described by Pollock *et al* we can assume that a DM phenotype has been maintained. If this is the case, then metabolic effects at a cellular level would not appear to influence the protein expression of Cx43 and Cx43(Ser368).

As has been described previously the development and recurrence of DFU is complex with multiple potential mechanisms affecting a foot at any given time. From the immunohistochemistry a pattern of Cx43(Ser368) expression has been demonstrated which seems to be associated with ischaemia. However infective processes are relevant and important in the development and progression of DFU. Consequently it was unclear whether the Cx43 and Cx43(ser368) expression was potentially being modified by an inflammatory environment. The influence of pro-inflammatory mediators on Cx expression and function has highly cell-specific effects. Cx43 protein expression is up-regulated [Garg 2005] in response to PGN in some cell lineages and down-regulated in others [Esen 2007]. At a cellular level GJ and CxHc function are also modified by PGN challenge, independent of changes in protein expression [Robertson 2010]. There is no similar work exploring the effect of PGN on Cx43(Ser368) protein expression, GJ or CxHc channel function.

The potential effects of a gram-positive inflammatory response on Cx43 and Cx43(Ser368) protein expression in human fibroblasts has been explored in vitro with PGN challenge, with up-regulation of Cx43 protein expression, but no change in Cx43(Ser368) protein expression. These data would support the assertion that the up regulation of Cx43(Ser368) protein expression is related to ischaemia. However the absence of similar data from human keratinocytes mean that definitive conclusion cannot be made. Furthermore infected DFU can represent a complex microbiological wound environment [Pellizzer 2001, Armstrong 2004] and gram-negative organisms may be relevant. The effects of lipopolysaccharides (LPS), which can be found on the outer membrane of gram-negative bacteria, have not been explored in the present work. Previous authors have demonstrated changes in Cx43 protein expression and phosphorylation in response to LPS challenge

[Liddington 2002, Liao 2010]. The effect of LPS on the expression of Cx43(Ser368) protein expression has not been explored.

Up regulation of Cx43(Ser368) protein associated with an acute wound seems to occur within 6 hours [Richards 2004, Pollock 2011]. The down regulation of Cx43 protein expression and up regulation of Cx43(Serine368) protein expression in the cardiac myocyte occurs quickly [Solan 2009, Pollock 2011, Johansen 2011]. In these experiments significant changes in Cx43 and Cx43(Ser368) protein expression have been demonstrated in vitro. Both Cx43 and Cx43(Ser368) protein expression were up regulated at 12 to 24-hours and 6 to 12-hours respectively with a reduction in protein expression thereafter. This would appear to be consistent with previously published data.

If this in vitro work is compared to a clinical vascular surgical presentation, this may be considered to be consistent with acute ischaemia (and is consistent with ischaemia/reperfusion models described by previous authors [Johansen 2011]). However, by definition all of the patients recruited to this study had chronic limb ischaemia. It may be that with longer periods of exposure to a hypoxic environment that there is a second peak in expression of Cx43(Ser368) protein. This merits further consideration and future investigation.

It is not clear whether skin expressing of Cx43(Ser368) protein is recoverable. In each of the cases where Cx43(Ser368) protein was demonstrated in vivo the limb was considered to be non-recoverable and major limb amputation was performed. Consequently the natural history (defined on clinical grounds) was for progressive tissue loss in these cases. From the present data it is unclear what the effect of revascularisation/re-oxygenation has on the expression of Cx43(Ser368) protein . Although there was ethical approval to perform skin biopsy on the study population if they re-presented for a digital amputation or foot debridement following successful arterial reconstruction this was not required for any of the recruited patients did. This could be explored in-vitro.

6.4 Unanswered questions and future investigation

In the first instance it would be important to validate the results presented here to justify the assertion that Cx43(Ser368) protein expression is a novel biomarker for ischaemia in skin. This would be best investigated in a group of patients undergoing either minor or major limb amputation, with a control group of patients having elective orthopaedic foot surgery.

In a future study, as well as assessing haemodynamic measures of perfusion the addition of transcutaneous oxygen pressure measurement may add useful information. If up regulation Cx43(Ser368) protein expression as a consequence of ischaemia in skin were validated defining the clinical utility/relevance would have to be assessed. Conceptually it may be informative in the context of a patient with digital ulceration requiring minor amputation (for extensive ulceration/sepsis). At the time of the procedure a skin biopsy from the healthy wound margin could be examined and if Cx43(Ser368) is demonstrated this may help inform decision-making with regard to arterial reconstruction if an option is available.

Whether skin biopsy could be justified from an ulcer margin (or elsewhere on a foot) that would not otherwise be managed surgically is not clear. There would have to be strong evidence from future study to justify this, given that there are a series of non-invasive investigations that can guide clinical decision-making.

Cx43(Ser368) protein expression could have a useful role as a research tool. For example the angiosome concept has been widely discussed with limited data (a relatively small number of single-centre retrospective studies) available to justify conclusion. If Cx43(Ser368) can be demonstrated to vary either qualitatively or quantitatively, a trial could be conducted recruiting patients admitted for major limb amputation with biopsies taken from each of the six-angiosome territories. The results could then be correlated with the pattern of PAOD that had been defined by the pre-operative cross-sectional arterial imaging.

There are also unanswered questions at a cellular level. Although conceptually this study did not seek to address these issues they are interesting and merit consideration and future investigation. Defining whether metabolic changes (hyperglycaemia/hypoglycaemia) influence the expression of Cx43(Ser368) protein is important if this protein is to be used

as a marker of ischaemia. Similarly identifying how Cx43 and Cx43(Ser368) protein expression are modified in response to re-oxygenation (in clinical terms re-perfusion following vascular surgical intervention) merits investigation. Finally defining how LPS influences Cx43(Ser368) protein expression is important if we are to define the specificity of this as a biological marker of ischaemia in skin.

Identifying the changes in GJ and CxHc expression, structure and functionality in ischaemic skin and the physiological/pathophysiological significance of these with regard to Cx43 and Cx43(Ser368) protein expression is essential if we are to better understand the effect and implications of these changes at a cellular level.

6.5 Conclusion

The complex pathophysiological processes that influence DFU make assessment challenging. Defining whether there is a significant ischaemic component as a consequence of PAOD relies on a constellation of clinical assessment, non-invasive investigation and radiological imaging. In isolation none of these can define the significance of PAOD and in this context a biological marker is an attractive concept.

Cx43 protein expression in skin sections appears to be consistent and the protein was not distributed differently in patients with significant ischaemia. Cx43 protein expression appears to be up regulated by hypoxia in vitro, but is also influenced by PGN challenge. As such these data suggest that Cx43 protein expression does not have utility as a biological marker of ischaemia in patients with PAOD..

Cx43(Ser368) protein expression appears to be up regulated in response to hypoxia in skin and consequently may have a role as a biological marker ischaemia. As with pre-existing investigative strategies it seems likely that this would be incorporated into a multi-modal investigative approach, rather than being a stand-alone investigation.

Further investigation is clearly required before a definitive conclusion can be made.

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Oviedo-Orta E, Kwak BR and Evans WH. Connexin Cell Communication Channels – Roles in the Immune System and Immunopathology

Appendix 1 – Solutions

Chemical reagents were obtained from Sigma Life Science or Fisher. Reagents for electrophoresis were obtained from Biorad.

Phosphate Buffered Saline (PBS) (Sigma Life Science)

One tablet dissolved in 200ml of distilled water (sterilised by autoclaving for 121°C for 15 minutes)

Protein Lysis Buffer

Prepared in RNase free eppendorf

1% sodium dodecyl Sulphate [SDS]; 1mM dithioreitol and 3nM sodium orthoranadate; in 870μl of PBS for a final volume of 1ml

Prior to use 10μl phenylmethylsulphenyl [PMSF] (100nM) and 10μl of protease inhibitor cocktail [PIC] were added

Tris-Buffered Saline and Tween 20 (TBS-T)

50 mM Tris

150 mM NaCl

0.1% Tween 20

Adjust pH with HCl to pH 7.4 - 7.6

SDS Running Buffer (10x Stock)

30g Tris

144g Glycine

10% SDS 100ml (made up to 1000ml with distilled water)

Protein Loading Buffer

1ml β-Mecaptoethanol

10% SDS

400μl Stacking buffer

2.5ml Glycerol

4ml Distilled water 2.1ml and Bromophenol Blue 0.1%)

Appendix 2 – Presentations and Published Abstracts

Hussey K, Kennon B and Stuart WP. **University of Texas diabetic ulcer classification the limbs at risk from presentation to a diabetic foot clinic.** British Journal of Surgery 2015; 102 (S2): 1-3

(Presented at the Vascular Society of Great Britain and Ireland ASM 2014 – British Journal of Surgery Prize Session)

Introduction

University of Texas (UoT) scoring is widely used to classify diabetic foot ulcers (a number indicates the ulcer depth and a letter describes the presence of sepsis and ischaemia). It has been validated as a predictor of outcome for ulcer episodes.

Methods

We examined our database to establish utility of this tool to predict the outcome for a limb (ulcer duration, major amputations and salvage procedures). The data were analysed by limb.

Results

Between 2005 and 2013 there were 971 ulcer episodes affecting 515 limbs (388 patients). UoT score implied peripheral arterial occlusive disease (PAOD) in 44.6% (UoT clinical groups C&D). Median duration of ulceration per limb was 4 months (range 1-63), significantly higher in the PAOD cohort compared with the non-arterial groups (A&B). There was also a significantly higher risk of major amputation (13.5 v's 4%) and. 21% of the PAOD limbs underwent salvage procedures (of which 31% still had a subsequent amputation), significantly more than in non-PAOD group (2%). In only12 limbs was the PAOD status upgraded from presentation.

Conclusions

UoT scoring robustly identifies the highest risk foot ulcers, indicating patients requiring intervention and those at highest risk of amputation. This may be a valuable tool for communication within the healthcare team.

Hussey K[1], Wright C[2], Martin P[2] and Stuart WP[1]. **Connexin 43 (Ser368) as a marker for ischaemia in diabetic foot ulceration.** BJS 2016; 103 (S5): 5–30

(Presented at the Vascular Society of Great Britain and Ireland ASM 2015 – British Journal of Surgery Prize Session)

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Introduction

The development and recurrence of diabetic foot ulceration (DFU) is multi-factorial. Identification of an ischaemic component can be difficult, given the indolent nature of neuroischaemic ulceration compounded by the presence of arterio-venous shunting and the frequently encountered distal pattern of arterial disease. We explored cutaneous expression of Connexin 43 (Ser368) *in-vivo* and in organotypic models of human skin.

Methods

Skin biopsies were performed on patients with and without diabetes undergoing arterial reconstruction or major limb amputation for critical limb ischaemia. Immunohistochemistry was performed using a primary Connexin 43 (Ser368) antibody. *In-vitro* assessment using human fibroblasts and keratinocytes cultured from skin was performed after subjecting the cells to hypoxic conditions. Protein expression was evaluated using Western blot analysis.

Results

Immunohistochemistry provided qualitative data demonstrating expression of Connexin 43 (Ser368) in skin biopsies harvested from ischaemic feet in all cases. Connexin 43 (Ser368) was not identified in any of the control specimens or in proximal skin biopsies of patients with occlusive arterial disease. When subjected to hypoxic laboratory conditions (5% oxygen) human fibroblasts and keratinocytes (from controls and study subjects) expressed Connexin 43 (Ser368) with protein expression peaking between 6 and 24 hours. These changes were not influenced by different glycaemic conditions.

Discussion

Cutaneous expression of Connexin 43 (Ser368) is a novel finding. These *in-vivo* findings, supported by organotypic modeling, suggest that Connexin 43 (Ser368) has potential utility as a biomarker for ischaemia in DFU.

Faniku C, Hussey K and Martin PE. **The impact of diabetes and ischemia on Connexin and Pannexin expression in the skin.** J Invest Derm 2016; 136(9): S257

A major problem of diabetic foot ulcers is ischemia contributing to delayed wound closure. The gap junction protein Connexin43 (Cx43) is differentially remodelled during 'normal' and 'chronic' wound healing events. The mechanisms by which this protein is involved remains unresolved. This work investigated the expression and post translational modification of Cx43 and Pannexin 1 (Panx1) in skin biopsies from patients undergoing arterial reconstruction or major limb amputation for critical limb ischaemia and in keratinocytes cultured in normoxic (N) and hypoxic (H) (1% oxygen, 5% carbon dioxide and 94 % nitrogen) conditions. Skin tissue biopsies (proximal and distal to the point of venous bypass surgery) from diabetic and non-diabetic patients were processed for immunohistochemistry and stained with antibodies specific to Cx43 and Cx43Ser^{368}. To model the events in vitro HaCaT cells were grown in 12 well plates, scrape wounded and cultured in N or H environments for up to 48 hours (h). Cell movement into the denuded area was recorded. Cells were fixed or protein harvested and expression of hypoxic inducible factor 1-a (HIF-1a), Cx43, Cx43Ser^{368}, Panx1 and Ki67 determined by immunocytochemistry and Western blot analysis. Cx43 and Cx43Ser^{368} expression was significantly increased in the epidermis of skin biopsies isolated from the distal position (i.e. locations of ischaemia) in both diabetic and non-diabetic tissue. Scratch wound assay determined delayed wound closure in hypoxic conditions, that was confirmed by induction of HIF-1a expression. Cx43 and Cx43^{Ser368} staining showed translocation of the protein to the nucleus. No changes in Panx1 were observed. In conclusion, post translational phosphorylation of Cx43 occurs in chronically wounded diabetic and ischemic skin. Under H conditions, scrape wound closure is reduced and immunofluorescence suggests that Cx43 and Cx43Ser^{368} translocate to the nucleus after 24 h hypoxia. Thus, hypoxia impacts on Cx expression but not Panx1 expression in the epidermis.